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Temperature-dependent development of porcine skeletal muscle cells in an *in-vitro* model

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Dedicated to my family

“Our fate lives within us. You only have to be brave enough to see it. “

(Merida Dun Broch)

Abstract (English)

Satellite cells are quiescent myogenic stem cells that are required for hypertrophic muscle growth and regeneration. It is possible to isolate satellite cells from muscle tissue and derive an *in vitro* model of these cells. This *in-vitro* model can be used to represent myogenesis using proliferating myoblasts and differentiating myotubes. It can be used to study the effects of various cultivation conditions on muscle development, such as temperature in the context of this thesis. Due to climate change and the associated occurrence of extreme temperature events, various cultivation temperatures are currently being investigation regarding the stimulation of myogenesis. Thermal stress can be triggered by both cold and heat and can affect farm animals' muscle growth, health, and welfare. This affects the animal food supply's safety and quality. The aim of the present PhD thesis is to establish a satellite-cell-derived primary muscle cell culture and investigate the effects of various cultivation temperatures on muscle cell growth in this *in vitro* model. Three studies were published for this purpose. The first study describes the comparative investigation of three muscle cell pools, along with their unpooled muscle cells. Muscle tissues taken from donor animals with various physiological characteristics can be used to establish an *in vitro* model. In piglets, the perinatal/early postnatal period is associated with high mortality, partially due to their immature thermoregulation. To account for the thermoregulatory properties of donor piglets, two cell pools were established using the *M. rhomboideus* of thermolabile 5-day-old (Pool 5) and thermostable 20-day-old piglets (Pool 20). No differences were found between the cell pools and their corresponding unpooled muscle cells regarding proliferative growth behavior; differentiation ability; and the mRNA expression of paired box 7 (PAX7), myoblast determination factor (MYOD), and myogenin (MYOG). The current results demonstrate that the use of cell pools is an appropriate method with which to reflect the average proliferative growth behavior of unpooled cells, and thus, the use of cell pools is adequate for the temperature experiments presented. Two subsequent studies investigated the effect of permanent cultivation below (35°C) and above (39°C, 41°C) the standard temperature of 37°C on the two muscle cell pools (Pools 5 and 20). All temperatures were found to be appropriate for the proliferation of porcine muscle cells, as no differences were found in their growth curves. However, temperature-dependent differences were found regarding these cells' biochemical and morphological properties, as well as their gene expression. Specifically, muscle cells cultured at 35°C are smaller and express more

of satellite cell marker PAX7's mRNA. These results suggest that they are inhibited in their myogenesis and still have the properties of activated satellite cells, as compared to myoblasts cultivated at higher temperatures. In addition, genes related to biological processes and signaling pathways, especially those involving the immune system (cytokine–cytokine receptor interaction, as well as the tumor necrosis factor (TNF) and interleukin-17 (IL-17) signaling pathways), were found which contribute to increased metabolic flux. When cultured at the physiological body temperature of piglets (39°C), as compared with 37°C, increased DNA content and differences in the expression of genes associated with DNA replication and cell growth were revealed. Despite similar growth characteristics, these differences indicate an adaptive mechanism and temperature threshold. Only cultivation at 41°C demonstrated a heat-stress response at the mRNA and protein levels, as well as the downregulation of many biological processes and signaling pathways related to proliferative ability. These cells were larger and expressed less of "early" muscle-specific transcription factors MYF5's and MYOD's mRNA, suggesting a more differentiated cell type as compared with that observed at 37 °C. The cell pools also reflect the different thermoregulatory properties of their donor piglets. In general, cells from Pool 5 (thermolabile) are smaller and express more PAX7 than those from Pool 20 (thermostable). The most transcription-related differences between the two muscle cell pools were found at the standard temperature of 37 °C, suggesting that both pools can access their full developmental potential at this temperature.

Abstract (Deutsch)

Satellitenzellen sind ruhende myogene Stammzellen, die für das hypertrophe Muskelwachstum und zur Regeneration benötigt werden. Es ist möglich, Satellitenzellen aus Muskelgewebe zu isolieren und aus ihnen ein *in vitro*-Modell abzuleiten. Anhand dieses Modells besteht die Möglichkeit, die Myogenese mithilfe proliferierender Myoblasten und differenzierender Myotuben widerzuspiegeln. Es kann verwendet werden, um die Auswirkungen verschiedener Kultivierungsbedingungen auf die Muskelentwicklung zu untersuchen, z. B. die Temperatur im Rahmen dieser Arbeit. Aufgrund des Klimawandels und des damit verbundenen Auftretens extremer Temperaturereignisse sind aktuell verschiedene Kultivierungstemperaturen zu untersuchende Stimuli der Myogenese. Temperaturbedingter Stress kann sowohl durch Kälte als auch durch Wärme ausgelöst werden und das Muskelwachstum, die Gesundheit und das Wohlbefinden landwirtschaftlicher Nutztiere beeinflussen. Dies hat wiederum Auswirkungen auf die Versorgung mit tierischen Lebensmitteln sowie auf deren Sicherheit und Qualität. Das Ziel der vorliegenden Doktorarbeit ist es, eine von Satellitenzellen abgeleitete primäre Muskelzellkultur zu etablieren und in diesem *in vitro*-Modell die Effekte verschiedener Kultivierungstemperaturen auf das Wachstum der Muskelzellen zu untersuchen. Dazu wurden drei Studien publiziert. In der ersten Studie wurde detailliert die vergleichende Untersuchung von drei Muskelzellpools mit deren ungepoolten Muskelzellen beschrieben. Für die Etablierung eines *in vitro*-Modells können Muskelgewebe von Spendertieren mit unterschiedlichen physiologischen Eigenschaften verwendet werden. Bei Ferkeln ist der perinatale sowie der früh-postnatale Zeitraum mit einer höheren Mortalität verbunden, was unter anderem an ihrer unausgereiften Thermoregulation liegt. Um die thermoregulatorischen Eigenschaften der Spenderferkel zu berücksichtigen, wurden zwei Zellpools aus dem *M. rhomboideus* von thermolabilen 5 Tage alten (Pool 5) und thermostabilen 20 Tage alten Ferkeln (Pool 20) etabliert. Es wurden keine Unterschiede zwischen den Zellpools und ihren korrespondierenden ungepoolten Muskelzellen für das proliferative Wachstumsverhalten, die Differenzierungsfähigkeit und die mRNA-Expression von paired box 7 (PAX7), myoblast determination factor (MYOD) und myogenin (MYOG) gefunden. Die aktuellen Ergebnisse belegen, dass die Verwendung von Zellpools eine geeignete Methode ist, um das durchschnittliche proliferative Wachstumsverhalten von ungepoolten Zellen widerzuspiegeln und somit die Nutzung von Zellpools für die

vorgestellten Temperaturversuche adäquat ist. In den beiden anschließenden Studien wurde die Auswirkung einer permanenten Kultivierung unterhalb von 35 °C und oberhalb von 39 °C sowie 41 °C der Standardtemperatur von 37 °C an den beiden Muskelzellpools 5 und 20 untersucht. Es konnte gezeigt werden, dass alle Temperaturen für Proliferation porciner Muskelzellen geeignet sind, da keine Unterschiede in ihren Wachstumskurven gefunden wurden. Allerdings wurden temperaturabhängige Unterschiede bei biochemischen und morphologischen Eigenschaften sowie bei der Genexpression gefunden. So sind bei 35 °C kultivierte Muskelzellen kleiner, und sie exprimieren mehr Satellitenzellmarker PAX7 mRNA. Diese Ergebnisse lassen darauf schließen, dass Myoblasten bei 35°C kultiviert in ihrer Myogenese gehemmt sind und im Vergleich zu Myoblasten bei höheren Kultivierungstemperauren noch Eigenschaften aktivierter Satellitenzellen tragen. Darüber hinaus wurden Gene gefunden, die mit biologischen Prozessen und Signalwegen zusammenhängen, insbesondere solche, die das Immunsystem betreffen (Interaktion zwischen Zytokin–Zytokinrezeptoren, tumor necrosis factor (TNF)- und interleukin-17 (IL-17)-Signalwegen), die auch zu einem höheren metabolischen Flux beitragen. Bei Kultivierung mit der physiologischen Körpertemperatur von Ferkeln von 39 °C konnten im Vergleich zu 37 °C ein höherer DNA-Gehalt und Unterschiede in der Expression von Genen, die mit der DNA-Replikation und dem Zellwachstum assoziiert werden, aufgezeigt werden. Trotz ähnlicher Wachstumseigenschaften weisen diese Unterschiede auf einen adaptiven Mechanismus und einen Temperatur-Threshold hin. Lediglich bei der Kultivierung mit 41 °C konnte eine Hitzestress-Antwort auf mRNA- und Protein-Ebene nachgewiesen werden sowie das Herunterregulieren zahlreicher biologischer Prozesse und Signalwege, die mit der Proliferationsfähigkeit zusammenhängen. Diese Zellen waren größer und exprimierten weniger ‚frühe' muskelspezifische Transkriptionsfaktoren myogenic factor 5 (MYF5) und MYOD mRNA, was für einen differenzierteren Zelltyp im Vergleich zu 37 °C spricht. Auch die Zellpools spiegeln die unterschiedlichen thermoregulatorischen Eigenschaften ihrer Spenderferkel wider. Generell sind die Zellen von Pool 5 (‚thermolabil') kleiner und exprimieren eine größere Menge an PAX7 als die von Pool 20 (‚thermostabil'). Die meisten Unterschiede zwischen den beiden Muskelzellpools wurden bei der Standardtemperatur von 37 °C gefunden, was darauf hindeutet, dass beide Pools bei dieser Temperatur ihr volles Entwicklungspotential abrufen können.

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CHAPTER 1

General introduction

1.1 Extreme temperature events

In its latest assessment report, the Intergovernmental Panel on Climate Change shows that the average temperature of the Earth's atmosphere and oceans has increased by about 1.1 °C from 1850 to 2020 (IPCC 2021). Additionally, simulations predict that, by 2100, the average global surface temperature will increase by about 5°C (Brown & Caldeira 2017, Meehl et al. 2020). This is associated with an increase in daily minimum temperatures, so the difference between the daily minimum and maximum temperatures will become smaller (Easterling et al. 1997). Furthermore, there was more land surface precipitation in the mid-latitudes, while this was absent in the tropics and subtropics, resulting in drought (Nicholls et al. 1995), and a change in extreme weather events is also expected (Meehl et al. 2000). An extreme weather event is an event that takes place at a certain time of the year and a certain place and is rare or extraordinary in this regard (DWD 2020). Additional attention is paid to so-called extreme temperature events, such as heat waves or cold spells. Since the beginning of the twenty-first century, multiple heat waves around the globe have occurred. Heat waves occurred in Europe in 2003 (Beniston 2004), in Russia in 2010 (Barriopedro et al. 2011), in India and Pakistan in 2015 (Masood et al. 2015, Ratnam et al. 2016), and South Korea in 2016 (Min et al. 2020). The consequences of such heat waves include a lack of rain, increasing the risk of forest fires and drought. In addition, cold spells are experienced in the following winter (Weilnhammer et al. 2021). Cities with warmer climates are more sensitive to cold spells, but this does not affect cities in the tropics or subtropics (Curriero et al. 2002). The increased frequency of such extreme weather events is undoubtedly due to anthropogenic activities associated with increasing industrialization and the accompanying emission of greenhouse gases (Trenberth 2012). The paradox is that climate change affects society, as well as the environment (Easterling et al. 2000). The scenario described above also reduces access to safe food and water, causes nutritional and water-borne diseases, and worsens pre-existing conditions (Macpherson 2014). The emission of greenhouse gases from agriculture also contributes to climate change and its associated consequences, such as extreme temperature events. These extreme temperature events are a major risk factor for the natural environment and agriculture productions systems, with implications for food security and prices (Campbell et al. 2016, Filho et al. 2022).

1.2 Relationship between extreme temperature events and animal production

Extreme temperature events are affecting crop and livestock production systems, as well as the stability of the food supply, food quality, and access to food (Schmidhuber & Tubiello 2007). In extreme temperature events, environmental factors, such as ambient temperature, humidity, and solar radiation, affect farm animals (Collier et al. 1982) and increase their risk of thermal stress. The commission for thermal physiology of the International Union of Physiological Sciences (IUPS, 2001) defines thermal stress as "any change in the thermal relation between an organism and its environment which, if uncompensated by temperature regulation, would result in hyper- or hypothermia." Therefore, thermal stress can occur due to cold or warm temperatures, and it affects farm animals' growth, development, health, and welfare, as well as the quality of the foods produced from them (Reed et al. 2017a).

Recently, simulation models have shown that the risk of cold spells may increase again from 2030 to 2040 (Mörner 2015, Lüdecke & Weiss 2017). However, the number of cold-stress-associated *in vivo* studies on farm animals is limited (Porter et al. 2022). Blood sampling of Atlantic salmon took place during the winter months (Sandnes et al.1988), and the influence of ambient temperature on thermolabile piglets has been investigated (Lossec et al. 1998a,b, Carrol et al. 2001). On the other hand, heat stress has always been of great importance in livestock production in the tropics and subtropics. In recent years, it has come into focus in the mid-latitudes due to global warming (Renaudeau et al. 2012, Sejian et al. 2018, Patra & Kar 2021). Meanwhile, *in vivo* heat-stress studies have been performed on all farm animals. Example include economically important fish species, such as salmon and trout (LeBlanc et al. 2012, Gamperl et al. 2021); ruminates, such as cattle, sheep, and goats (Koch et al. 2019, Chauhan et al. 2014, Dangi et al. 2016); and poultry, such as chicken and turkey (Bartlett & Smith 2003, Rozenboim et al. 2004). A large number of *in vivo* studies of pigs have investigated the effects of heat stress on their physiology (Le Bellego et al. 2002, Patience et al. 2005, Hao et al. 2014) by using proteomic (Cruzen et al. 2015) or epigenomic analysis (Hao et al. 2016) or by focusing on gestational period (Johnson et al. 2015). In addition, the effect on feeding behavior on fattening pigs has also been investigated, showing that reduced feed intake leads to increased resting behavior, reduced feeding time, and decreased *ad*

libitum portion size (Hicks et al. 1998, Brown-Brandl et al. 2001, Collin et al. 2001). In addition, Cross et al. (2018) found genetic differences in the feeding behaviors of fattening pigs between higher ambient temperatures and lower ambient temperatures. Additional consequences of heat stress include increased body temperature, respiration rate, and heart rate, while fecal and urinary water loss and feed intake are decreased. This alters the energy-partitioning metabolism and, ultimately, affects growth rates, reproductive performance, and carcass quality (Baumgard & Rhoads 2013, Pearce et al. 2013).

Moreover, heat stress is known to be associated with pale, soft, and exudative (PSE) meat in pigs (Gonzalez-Rivas et al. 2020), as well as with dark, firm, and dry (DFD) meat in beef cattle and small ruminants (sheep and goat; Gregory 2010). Traditionally, the belly, ham, and loin comprise the 75% of a carcass's economic value, and therefore, any factors that alter these components will produce economic losses (Marcoux et al. 2007). Pigs reared under heat-stress conditions exhibited increased lipid storage (Pearce et al. 2013, Qu & Ajuwon 2018) via enhanced lipid metabolism in the adipose tissue of lipoprotein lipase (LPL) activity and hepatic very low density lipoprotein (VLDL; Kouba et al. 2001, Qu et al. 2016). Several proteomics studies have found an association between the expression levels of heat shock proteins (HSPs) and meat quality traits (Hwang et al. 2005, Sayd et al. 2006, Bernard et al. 2007, Kim et al. 2008, Pulford et al. 2008, Pulford et al. 2009). Heat shock proteins are important components of living muscle, modulating the cytoskeleton and controlling cell maintenance and repair (Carra et al. 2017). The downregulation of the expression of HSPs is associated with improved muscle tenderness, juiciness, and flavor (Bernard et al. 2007). The upregulation of HSPs during heat stress can, therefore, be associated with reduced beef tenderness by altering the protein folding, polymerization, and aggregation of microtubules, as well as retarding cellular death during the conversion of muscle to meat (Bernard et al. 2007, Lomiwes et al. 2014, Cruzen et al. 2015).

1.3 Thermoregulation in pigs

Thermoregulation is defined as the balance between heat production and heat loss mechanisms, which are intended to maintain a relatively constant body temperature (Renaudeau et al. 2012). Pigs, as mammals, are homoeothermic, as evidenced by their stable body temperature. In addition, the maintenance of a high, constant body temperature involves the delicate balance between heat production and heat loss (Collier et al. 2019, Gourdine et al. 2021). Physiological thermoregulation matures during ontogeny, and this function must be established in nest-hocking young animals (Tembrock 1982).

The piglet, unlike other newborn mammals, has no brown adipose tissue (BAT); therefore, piglets are poorly insulated and do not have a stable body temperature (Symonds & Lomax 1992). Regarding the maintenance of their homoeothermic equilibrium, the skeletal muscles play an essential role (Berthon et al. 1994, Fuller-Jackson & Henry 2018). In addition, their larger body-mass-to-surface ratio results in a higher amount of heat loss. During gestation, the fetal body temperature is strongly linked to the intra-uterine environment (Herpin et al. 2002). At birth, body temperature drops by 2°C within the first 20 min due to a rapid decrease in the environmental temperature. It can take up to 48 h until the physiological body temperature of 39°C is reached, and a stable body temperature is manifested after the first week of life (Mount 1968, Curtis & Rogler 1970, Berthon et al. 1993). Within the first 24 h postpartum (p.p.), the piglets typically lie close to the sow (Titterington & Fraser 1975), and a reduction of heat loss is achieved by behavioral adjustments, such as huddling with littermates (Hrupka et al. 2000a, b). This shows that the early postnatal period is very crucial for the further fate of the piglet's life, and this period has the highest mortality rate (Tuchscherer et al. 2000). Therefore, changes in the neonatal thermal environment, such as cold stress, should be avoided (Becker et al. 1997). Cold stress and infectious disease are suspected to contribute to the 13 to 15% mortality rate reported for piglets between farrowing and weaning (Carrol et al. 2001). The industry standard for the lower crucial temperature (LCT) of 34.6°C at 2 h of age in neonatal pigs was derived from Herpin et al. (2002). In addition, a study of postnatal piglets kept at an ambient temperature of 18°C and subsequently subjected to an immune challenge via lipopolysaccharides (LPS) showed that these piglets coped with LPS more poorly and their body temperature dropped more

sharply as compared to a control group (Carrol et al. 2001). Providing this supplemental heat not only improves piglet performance but may also be essential for the proper functioning of the immune system and survival (Carrol et al. 2012). Producers often offer supplemental heat in the form of heat pads and heat lamps to maintain environmental temperatures above the LCT for neonatal pigs.

There are two main mechanisms for heat production: shivering and non-shivering thermogenesis. Newborn piglets shiver with a very high intensity, which is because piglets have very little adipose tissue (1.5%) as compared to other newborn mammals and do not possess BAT (Mount 1986, Herpin et al. 2002). Until weaning, this situation changes very quickly, with piglets achieving a fat content up to 15% (Dauncey et al. 1981). Brown adipose tissue is essential for non-shivering thermogenesis in the form of heat production via uncoupling oxidative phosphorylation. The key marker of this process is uncoupling protein 1 (UCP1), which is exclusively expressed in BAT. In pigs, exons 3–5 have been deleted, and the remaining exons of this six-exon gene each display an inactivating (frameshift, insertion/deletion, or nonsense) mutation (Fyda et al. 2020) and cannot generate heat via non-shivering thermogenesis.

Depending on the postnatal intensity of shivering, piglets can generate heat via shivering thermogenesis. The heat power of this process increases with postnatal age because of the increased electrical activity of shivering (Berthon et al. 1994). Skeletal muscle and its energy metabolism play in important role in this process. To produce heat, the muscle fibers must achieve an optimal balance between excitation and contraction. The contraction power of the muscle fibers is linked to the myofibrils' mass, but after birth, piglets' fibers lack myofibrils and their volume increases rapidly during the first 5 days p.p. (Bradley et al. 1980, Handel & Stickland 1987). In addition, for optimal power contraction, an adequate supply of substrates and oxygen is needed to synthesize ATP, as shown in **Figure 1.1** (Herpin et al. 2002). Muscle blood flow is essential for oxygen and nutrient supply. Lossec et al. (1998b) showed that blood flow of 5-day-old, as compared to 1-day old, piglets under cold conditions is three times higher, suggesting an improved blood supply to the skeletal muscle during the first days of life. In addition, the mitochondrial mass strongly increased in oxidative (more than 90%) and glycolytic (nearly 50%) skeletal muscles within this timeframe. This was accompanied by optimal ATP supply via oxidative phosphorylation, which is required during shivering.

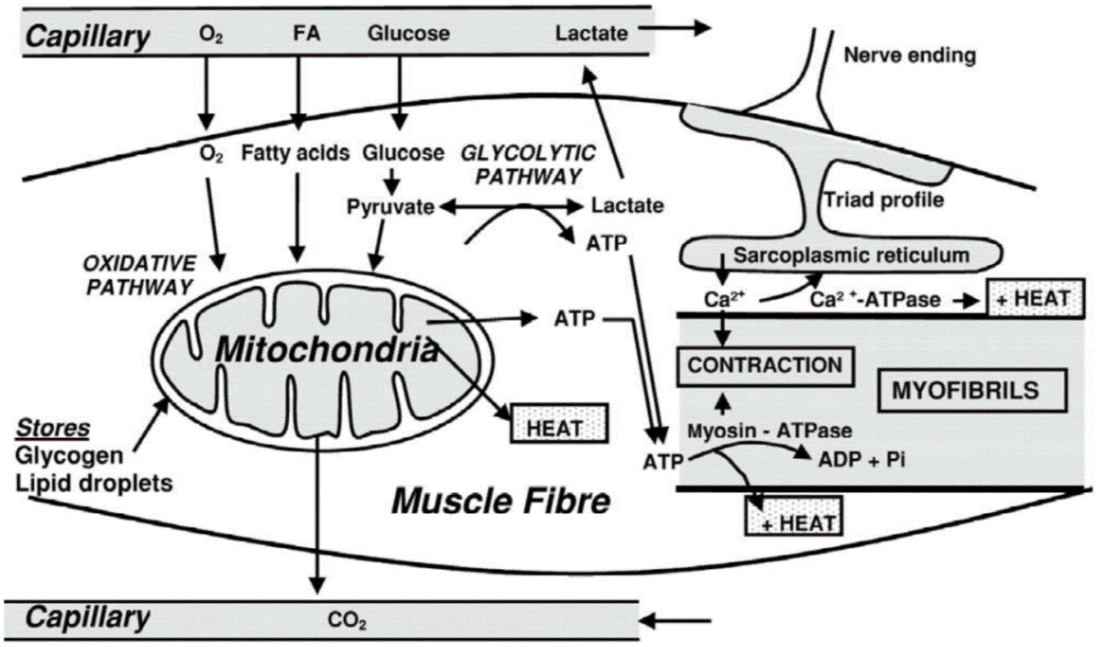

Figure 1.1: Overview of structures and pathways involved in shivering thermogenesis modified from Herpin et al. 2002. The capillaries supply the muscle with oxygen, fatty acids, and glucose (pyruvate). These were metabolized by mitochondria to generate energy in the form of ATP. During this process, heat is also generated. The resulting ATP is used by myosin for contraction to generate heat.

When piglets have reached their full thermoregulatory capacity, they have a stable average body temperature of 38.8°C (Ingram & Legge 1970). Thermoregulation has an influence on the housing system. For example, in free-range or outdoor farming, precautions must be taken to protect the pigs from thermal stress during extreme weather events (Ludwiczak et al. 2021, Wimmler et al. 2022).

1.4 Skeletal muscle development in pigs

Skeletal muscle contributes to vital functions such as movement, postural support, breathing, and thermogenesis (Dumont et al. 2015). In addition, it contribute to energy metabolism by serving as a storehouse of amino acids and carbohydrates, contributing to heat production to maintain a stable body temperature, and consuming a large share of oxygen and other substances during physical activity (Frontera & Ochala 2015). Skeletal muscle is a syncytium and consists of muscle fibers embedded in a connective tissue that is continuous with the tendon (**Figure 1.2**). At the edge of the muscle fibers, there are numerous myonuclei that are relatively constantly distributed, with approximately 2 x 10^4 to 5 x 10^4 myonuclei/μm^3 (Dumont et al. 2015). They are embedded by common cytoplasm, which is filled with myofibrils and is composed of serially connected sarcomeres. The interaction between the myofibrillar proteins actin and myosin generates force and, thereby, shortens the sarcomeres. Myosin is composed of myosin heavy chain (MYHC), which determines the contraction properties of myofibers depending on their isoforms, with different levels of ATPase activity (Zammit 2017). In pigs, slow-twitch type I, fast-twitch type IIa, and fast-twitch type IIb are contractile fiber types, and red (oxidative), intermediate, and white (glycolytic) are metabolic fiber types (Rehfeldt et al. 2008). However, a contractile fiber type may well be assigned to different metabolic fiber types (Essén-Gustavsson & Lindholm 1984). The new myonuclei required for homeostasis, hypertrophy, and repair/regeneration are supplied from a population of resident stem cells, the so-called satellite cells (Zammit 2017). Satellite cells are located between the sarcolemma and basement membrane of muscle fibers (Mauro 1961). Paired box 7 (PAX7) is the marker for satellite cells (Seale et al. 2000), and it belongs to a family of genes that encode paired box-containing transcription factors involved in the control of developmental processes (Jostes et al. 1991, Schäfer et al. 1994).

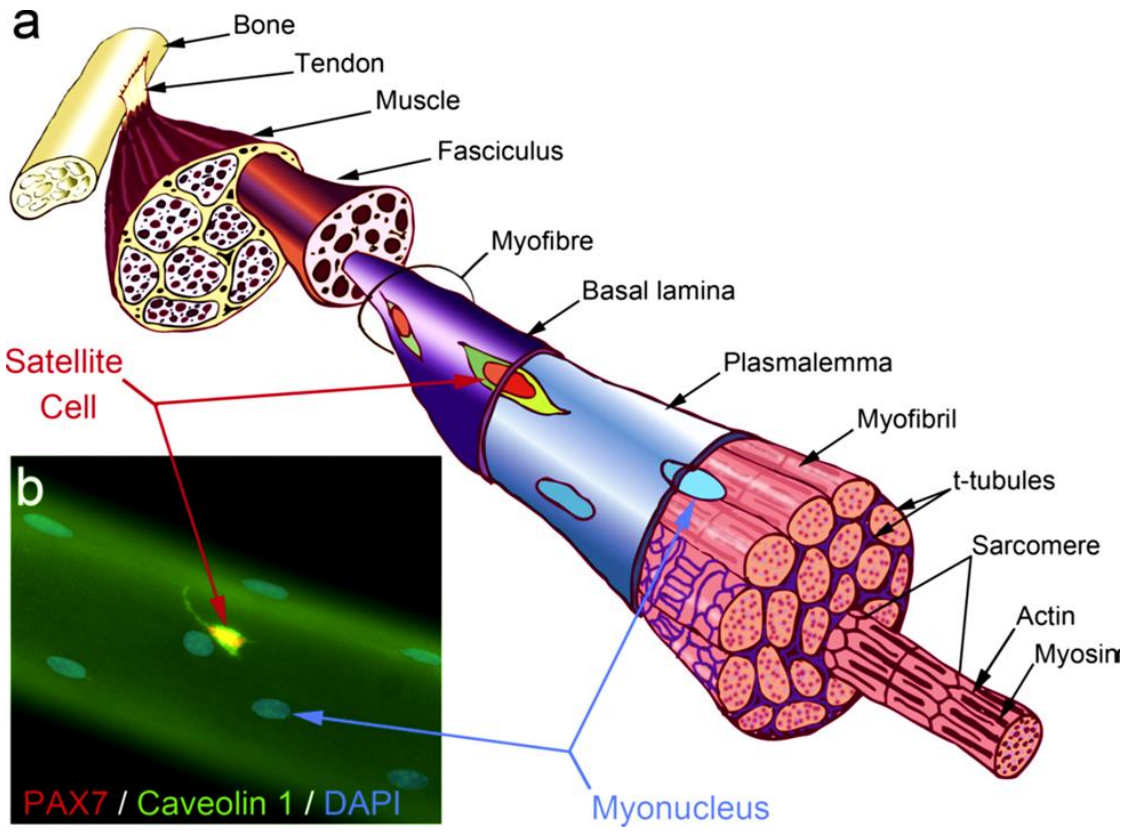

Figure 1.2: Muscle structure and satellite cell modified from Zammit (2017). Schematic showing the structure of skeletal muscle. The satellite cell niche is on the plasmalemma of the myofiber, beneath the surrounding basal lamina (a). A quiescentmurine satellite cell retained in its niche on a myofiber isolated from the extensor digitorum longus muscle of an adult mouse (b).

The most important muscle fiber organelle for energy metabolism is the mitochondria. They generate the energy required by muscle in the form of adenosine triphosphate (ATP). The main appropriate sources of energy for muscle ATP production are carbohydrates (plasma glucose and muscle glycogen) and fats (free plasma fatty acids and muscle triglycerides; Romjin et al. 1993). There are three major systems responsible for ATP resynthesis: the phosphagen system, the glycolytic system, and mitochondrial respiration (Hocquette et al. 1998, Baker et al. 2010). Mitochondrial respiration occurs in the inner membrane of the mitochondria, and this where the actual ATP-formation process takes place. It is also closely interrelated with the tricarboxylic acid (TCA) cycle. The reduced nicotinamide adenine dinucleotide ($NADH + H^+$) produced there flows into the first complex, and the hydroquinone form of flavin adenine dinucleotide ($FADH_2$) flows into the second complex. In the last step of the respiratory chain, in the fourth

complex, electron transfer to oxygen takes place (Stangl 2011). As mentioned in Chapter 1.3, energy metabolism contributes significantly to thermoregulation, and it would be interesting to investigate how this highly complex system adapts to an external temperature stimulus. Recently, Little and Seebacher (2016) have shown that murine C2C12 myoblasts grown at 32°C exhibited significantly increased rates of basal oxygen consumption, maximal oxygen consumption, proton leak, net ATP production, and anaerobic metabolic rates, which are markers of mitochondrial stress. However, the effect of a temperature below and above the standard cultivation temperature has not yet been investigated in a primary muscle cell culture.

Myogenesis is the formation and development of skeletal muscle. The myogenic processes and regulation by a coordinated transcriptional hierarchy involved in myogenesis are of interest. During the earlier embryogenesis, somites develop from the paraxial mesoderm and have an epithelial structure. Somites are important in organizing the segmentation pattern of vertebrate embryos. In the dorsal part is the dermomyotome, which matures into the dermotome and myotome. The myotome is used for skeletal muscle development, and the pluripotent cells located there become myoblasts (Brent & Tabin 2002, Picard et al. 2002, Bentzinger et al. 2012).

Embryonic myogenesis is a multistep process that begins with embryonic myogenic precursor cells or progenitor cells. The embryonic progenitor cells proliferate into embryonic myoblasts, followed by the differentiation of myoblast into myocytes and, finally, fusion into multi-nucleotated myotubes. The mature myotubes form primary fibers. During fetal growth, the fetal myoblasts go through this process, and the resulting secondary fibers are assembled around the primary fibers. However, in large mammals (e.g., sheep and pigs), tertiary fibers have also been described, which develop between the secondary fibers. Throughout these developmental stages, hyperplasia occurs after the formation of the secondary fibers. Further hypertrophic growth, regeneration, and repair originate from the satellite cells (Lefaucheur et al. 1995, Bérard et al. 2011, Knight & Kothary 2011, Rehfeldt et al. 2011, Bentzinger et al. 2012).

The cellular processes of muscle development and differentiation are comparable in all species, but the time course is species specific. Myogenesis in pigs begins with the formation of primary muscle fibers at 35 days post-conception (dpc) and lasts until 60 dcp. Subsequently, the secondary muscle fibers are formed; they use the primary muscle

fibers as a scaffold and wrap themselves around them. The formation of the secondary muscle fibers takes place between 55 and 90 dpc (Wigmore & Stickland 1983). At the time of birth, the satellite cells are formed. In addition, the formation of tertiary muscle fibers takes place from shortly after birth to up to 3 weeks of age (Mascarello et al. 1992, Lefaucheur et al. 1995, Bérard et al. 2011). In pig skeletal muscle, the total fiber number and fiber type composition influence postnatal growth performance, carcass characteristics, and meat quality (Rehfeldt et al. 2000, Lefaucheur et al. 2002, Bee 2004, Bérard et al. 2011).

A group of transcription factors has the ability to induce myogenesis in various cell types, which is why, together, they form the myogenic regulatory factor family (Weintraub et al. 1991). The myogenic regulatory factors (MRFs) are subdivided into early myogenic factors like myogenic factor 5 (MYF5) and myoblast determination factor (MYOD), which are involved in early myoblast determination, and late factors like myogenin (MYOG) and myogenic regulatory factor 4 (MRF4), which promote differentiation (Bentzinger et al. 2012). These MRFs' protein structures are highly related to one another. These basic helix-loop-helix (bHLH) transcription factors contain three conserved domains: the amino terminal transactivation domain, with a histidine/cysteine-rich zone; the central region, with the bHLH motif, including the α-helical basic domain and Helix I and II; and another transactivation domain in the carboxyl terminal, containing Helix III (Singh & Dilworth 2013). The expression of MRFs is linked to the fate of activated satellite cells. The activated satellite cells become a myoblast, and the expression of MRFs begins. MYF5 and MYOD are mainly involved in muscle specification, while during adult myogenesis, an activated satellite cell first expresses MYF5 and promotes their proliferation. Subsequently, MYOD is expressed and induces a withdrawal from the cell cycle. With the beginning of myocyte formation, the expression of MYOG starts. MYOG is the key factor affecting myoblast differentiation. Finally, MRF4 is involved in the maturation of myocytes into myofibres (Le Grand & Rudnicki 2007, Bentzinger et al. 2012, Zanou & Gailly 2013). Specific proteins, including the regulatory wingless proteins (Wnt) and insulin like growth factors (IGFs), and specific genes, such as PAX3 and 7, have been found to control the expression of MRFs (Knigth & Kothary 2011).

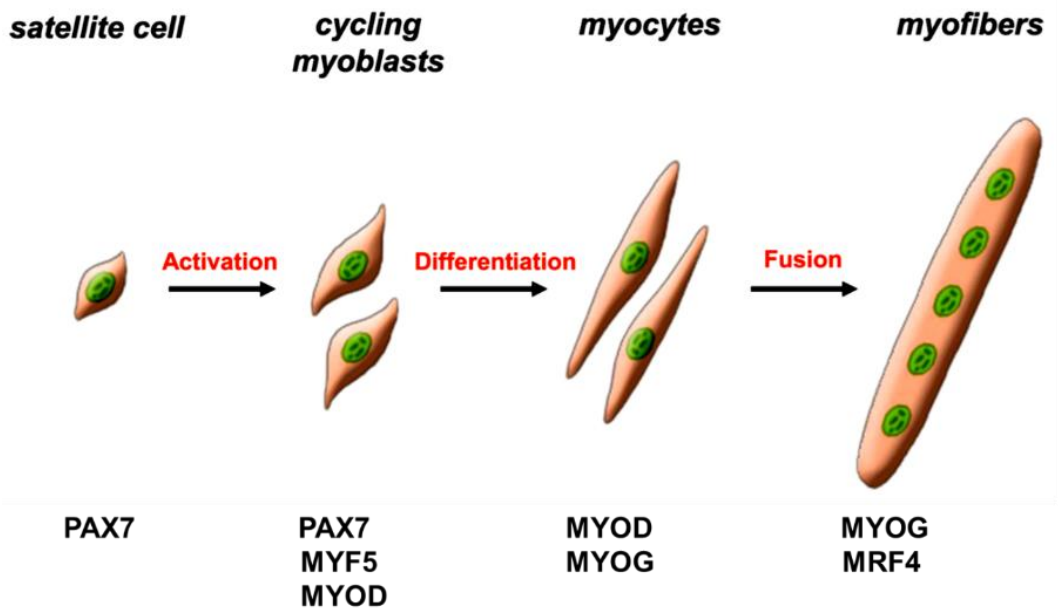

Figure 1.3: Schematic representation of adult myogenesis (modified from Le Grand & Rudnicki 2007). Satellite cells are quiescent myogenic stem cells. After their activation, myogenic factor 5 (MYF5) is expressed, and proliferation starts. While myoblast determination factor (MYOD) is involved in myoblast differentiation, myogenin (MYOG) is involved in myoblasts' fusion into a myofiber, and myogenic regulatory factor 4 (MRF4) is involved in myofiber maturation.

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1.5 Satellite cells as an *in vitro* model

In 1961, a paper by Mauro was published that identified a cell on the surface of the skeletal muscle fiber via electron microscopy. This cell was called a satellite cell, uniting the name myosatellitocytus with the defining anatomical location (Mauro 1961). Satellite cells are sparsely distributed in the muscle (with 1–4% of muscle nuclei in fast-twitch muscles and three or four times more in slow-twitch muscles; Bischoff 1994). There are two *in vitro* culture systems associated with satellite cells: the single-muscle-fiber cultures and satellite cell cultures derived from digested skeletal muscle, both of which are isolated directly from skeletal muscle (Allen et al. 1998). Working with a single-fiber culture offers the advantage that satellite cells can be maintained in a quiescent state and their characteristic position. The isolation of single muscle fibers was first performed using rat skeletal muscle (Bekoff & Betz 1977, Bischoff 1986) and, later, with goat (Yamanouchi et al. 2009) and pig (Wilschut et al. 2010) muscle fibers. Alternatively, working with primary satellite cell cultures derived from digested skeletal muscle tissue offers the advantage that a large number of satellite cells can be generated. Regarding the three Rs (reduce, replace, and refine), working with an *in vitro* culture system is an alternative method and, consequently, a replacement (Russel & Burch 1959). Cell cultures can be performed using one individual animal or as a cell pool consisting of cells from several animals. However, cell pooling is hotly debated because the specificity of muscle-derived cultures reflects the physiological and developmental states of the donors. Therefore, donor selection should consider age, muscle mass, disease or injury, endocrine status, and other physiological conditions (Allen et al. 1998, Stoddart et al. 2012). Still, it is generally agreed that cell pooling offers an opportunity to adopt long-term approaches with a broad spectrum of experiments and analyses, as well as a large number of replications. Porcine muscle cell pools have been established from newborn and 5-day-old donor animals and derived from one muscle (Mau et al. 2008b, Zhao et al. 2021), and multiple pools have been created from different muscle tissues from the same newborn donor animals (Will et al. 2012). Porcine muscle cell pools from donors with different thermoregulatory maturity levels have never been established before. Therefore, working isolated satellite cells derived from the *M. rhomboideus* of thermolabile piglets (5 days of age) and thermostable piglets (20 days of age) is a new approach.

The first isolation protocol for satellite cells was done with rat muscles and was created by Bischoff in 1974. In 1992, Doumit and Merkel published an adapted protocol for pig muscles. To the present day, isolation protocols exist for all relevant agricultural species, like sheep (Dodson et al. 1986), cattle (Dodson et al. 1987), chicken (Yablonka-Reuveni et al. 1987), turkeys (McFarland et al. 1988), fish (Powell et al. 1989), and horses (Greene & Raub 1992). After isolation, satellite cells can be cultivated as proliferating myoblasts or differentiating myotubes. Therefore, they provide a suitable model for muscle biology research via the adult myogenesis (**Figure 1.3**) that occurs after the activation of the satellite cells. Because of the similar programmed transcriptional profiles between embryonic and adult myogenesis (Parker et al. 2003), primary satellite cell cultures can be a suitable model for embryonic myogenesis. This approach is also referred to as "development in a dish" (Chal J. & Pourquié 2017).

Furthermore, this cell model enables the investigation of the direct effect of bioactive substances, such as phenolic compounds (Bhattacharya et al. 2013) or fatty acids (Che et al. 2013), on muscle growth and differentiation. In addition, external stimuli, such as temperature, can be investigated by changing the cultivation temperature. Studies on the effects of different cultivation temperatures on human muscle cell cultures (range of 37°C to 41°C; Yamaguchi et al. 2010) and avian muscle cell cultures cultivated below and above 38°C exist (chicken: Harding et al. 2015, Harding et al. 2016; turkey: Clark et al. 2016, Clark et al. 2017, Reed et al. 2017a,b, Reed et al. 2022a,b). Only two studies of porcine muscle cell cultures under heat stress conditions have been performed (Kamanga-Sollo et al. 2011, Gao et al. 2015). Both studies used porcine muscle cell cultures with a single temperature challenge of 40.5°C or 41°C for only hours, whereas permanent cultivation was performed at 37°C. For a deeper insight, it would also be of importance to study the effect of different cultivation temperatures on the transcriptome. Previously, only a few studies have investigated the effect of different cultivation temperatures on the transcriptome. One deals with an immortal mouse muscle cell line, C2C12, and the others deal with turkey muscle cell cultures. The C2C12 cells were cultivated above and below the standard cultivation temperature of 37°C for 72 h. Myoblasts cultivated below the standard cultivation temperature differently expressed the genes involved in enriched pathways, including the phosphatidylinositol 3-kinase (PI3K) - protein kinase B (Akt) pathway, the lysosome, hypoxia-inducible factor (HIF) signaling, and wingless proteins (Wnt) signaling, which were downregulated. The same pathways were also detected in a

cultivation performed above 37 °C and found to be upregulated (Risha et al. 2022). The turkey muscle cells were cultivated at 33°C, 38°C (control), or 43°C until myoblast proliferation or myotube differentiation. After proliferation, myoblasts cultured at 33°C exhibited an overrepresentation of genes involved in cell signaling/signal transduction as compared to the myoblasts cultered at the control temperature. Cultivation at 43°C led to genes related to muscle system development and differentiation exhibiting increased expression levels (Reed et al. 2017a). For myotubes, cultivation at 33°C resulted in the downregulation of genes assigned to the regulation of skeletal muscle tissue regeneration, sarcomere organization, and the regulation of Ca^{2+} homeostasis as compared to cultivation at 38°C. In contrast, cultivation at 43°C, as compared to 38°C, resulted in the upregulation of genes assigned to regulating myoblast differentiation and survival and cell adhesion (Reed et al. 2017b, Reed et al. 2022b). With regard to the occurrence of extreme temperature events, which can occur in either warm or cold periods, a gap exists today. There are no studies using porcine muscle cell cultures that have been permanently cultured above or below the standard cultivation temperature regarding the influence of such on the transcriptome.

1.6 Objectives of this thesis

Due to the occurrence of extreme temperature events, which manifests itself in cold spells as well as in heat waves, there is a risk for the environment and the agriculture. This result in thermal stress in farm animals and has an impact on their health, welfare, and growth as well as productivity. The myogenesis is the basic process for muscle development and growth and a prerequisite for the quality of meat. To study thermal effects on myogenesis *in vitro* an appropriate model was established using cell pools derived from satellite cells of piglets with different thermoregulatory maturities. Piglets are born without brown adipose tissue and their thermoregulation mainly occurs in skeletal muscle. It needs the first week of life that piglets could maintain their own body temperature. Therefore, muscle cell pools from piglets at the age of five or twenty days were established as thermolabile or thermostable *in vitro* models, respectively. This approach is novel and enable to compare the thermoregulatory maturity of the donor piglets. Previous studies with porcine muscle cell cultures aimed to induce heat stress by a punctual stimulus in form of a single temperature challenge above 40°C. To the best of our knowledge, studies on adaptation of porcine muscle cell cultures on a moderate but permanent cultivation above and below the standard cultivation temperature are missing. Therefore, the aim of this thesis was to establish two porcine muscle cell pools from piglets with different thermoregulatory maturities and characterize their permanent cultivation below (35°C) and above (39°C, 41°C) the standard cultivation temperature of 37°C during proliferative growth (Figure 1.4).

The objective of the study is this to investigate the following hypotheses:

Whether the cell pool-based approach is representative compared to individual samples and the advantages and disadvantages of this approach.

In **Chapter 2**, three different muscle cell pools and their corresponding unpooled cells from donor animals, which differed in terms of the muscles chosen, gender, and age, were used. Proliferative growth was monitored in real time and after 72 h, the mRNA expression levels of PAX7, MYOD, and MYOG were analyzed. Furthermore, the muscle cells were differentiated, and the fusion degree was determined.

Whether prolonged exposure to temperatures between 35 °C and 41 °C, with 2 °C incremental temperature increases, are suitable for the cultivation of proliferating myoblast was investigated.

Both porcine muscle cell pools were permanently cultured for 72 h at 35 °, 37 °, 39 ° or 41 °C, where 39 °C corresponds to the physiological body temperature of pigs. Real-time impedimetric monitoring was used to evaluate the proliferative growth behavior of myoblasts in **Chapter 3**.

Whether cultivation temperatures and donor cells representing differences in thermoregulatory capacities have an impact on physiological relevant parameters, signaling pathways, or energy metabolism.

After proliferative growth morphological and biochemical properties of myoblasts were analyzed. The hypothesis-based gene expression levels of myogenic regulatory factors, muscle-associated genes, and heat shock proteins were determined in **Chapter 3**. Microarray analysis, as a modern omics technique, was used to identify the temperature-dependent transcript profile.

In addition, energy metabolism and the oxidative status were analyzed in **Chapter 4**. All experiments were done in parallel, with two muscle pools of 5-day old piglets (thermolabile) and 20-day old piglets (thermostable). Various physiological and energy metabolism parameters, as well as transcript profiles, were described in additional sections of **Chapters 3 and 4**.

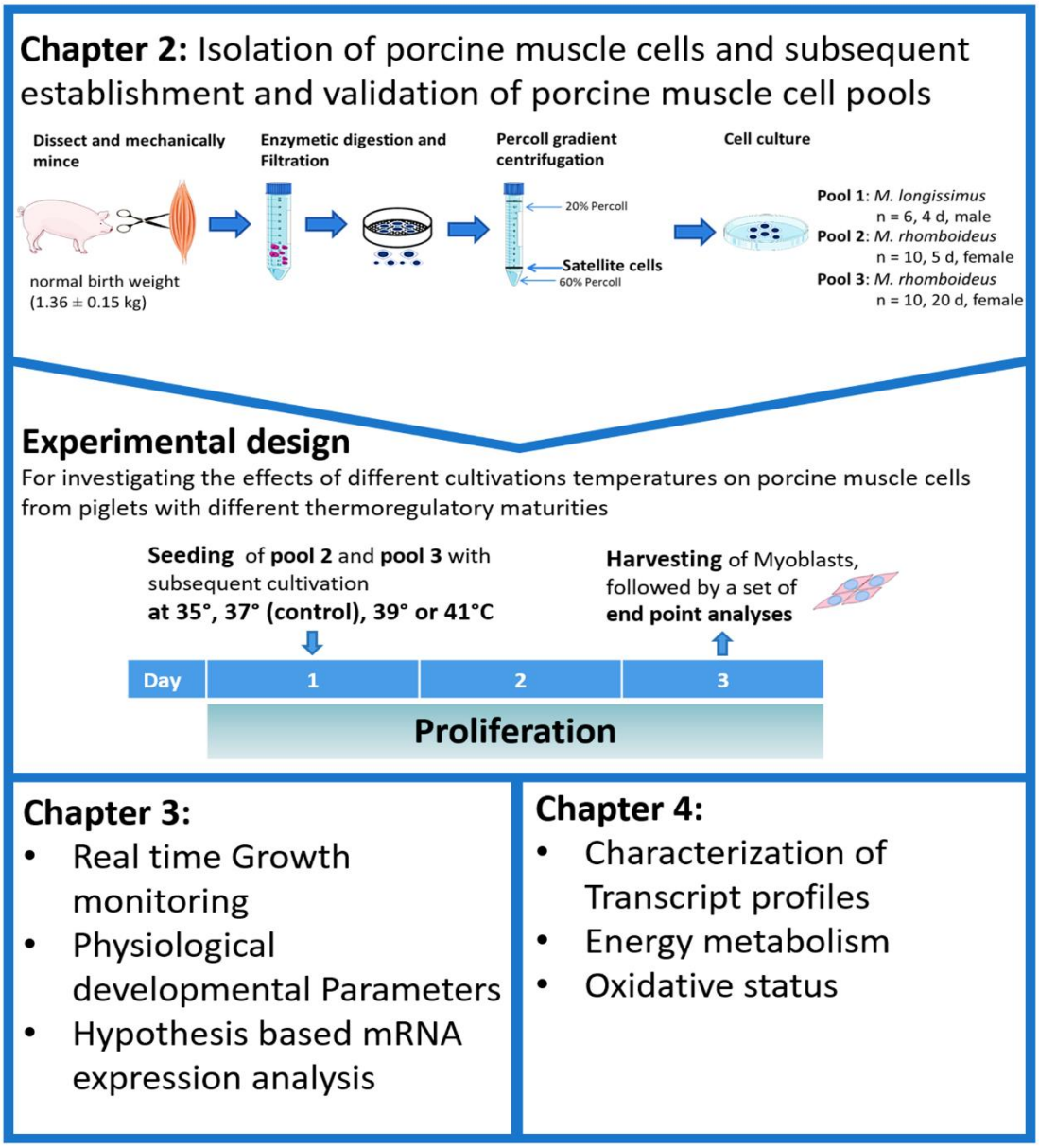

Figure 1.4: Schematic overview of chapters that are part of this thesis.

CHAPTER 2

Based on

Establishment and validation of cell pools using primary muscle cells derived from satellite cells of pig skeletal muscle

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Establishment and validation of cell pools using primary muscle cells derived from satellite cells of pig skeletal muscle

Abstract

Primary cell cultures derived from satellite cells of skeletal muscle provide an appropriate *in vitro* model for proliferating myoblasts and differentiating myotubes for muscle biological research. These cell cultures may consist of harvested cells per animal or of a cell pool made of cells from several animals. However, cell pooling reduces the biological variability of the different cell donors. On the other hand, the use of cell pools offers an opportunity to use less donor tissue and to perform long-term projects with a broad spectrum of analysis and replications. In the literature, information about the donors of cell pools, the procedure used for pooling and the characterization/validation of cell pools is often lacking. In this study, we established three cell pools consisting of *M. rhomboideus* or *M. longissimus* from ten or six piglets, each with one gender and medium birth weight. Real-time impedimetric monitoring was used to evaluate the proliferative growth behavior of myoblasts for the cell pools in comparison to their corresponding unpooled cells over a period of 72 h, with a measurement being taken every 30 min. For each of the tested cell pools, cell index, slope and doubling time did not differ between the cell pool and the unpooled cells of the donor animals. Differentiation capacity and mRNA expression of *PAX7*, *MYOD* and *MYOG* remained unchanged between the cell pool and the unpooled cells. Current results support that the use of cell pools is an appropriate method to reflect the average proliferative growth behavior of unpooled cells.

Key words

Cell pool, satellite cells, myoblasts, growth profile, real-time monitoring

The discovery of satellite cells (Mauro 1961) from skeletal muscle, their isolation and their subsequent cultivation as proliferating and differentiating progenies (myoblasts and myotubes) provides a unique model for muscle biology research. It is known that the adult myogenesis occurring after the activation of satellite cells can be viewed as a suitable model for embryonic myogenesis and that their programmed transcriptional profiles are similar (Parker et al. 2003). Moreover, these in vitro systems enable research investigating the direct effects of bioactive compounds (e.g., elderflower extracts (Bhattacharya et al. 2013), phytoestrogens (Kalbe et al. 2008) or fatty acids (Mc Farland et al. 2011)) on muscle growth and differentiation.

There are two different approaches to isolate satellite cells from skeletal muscle: the direct isolation of satellite cells from digested muscle tissues and the isolation of single muscle fibers. The direct isolation of satellite cells yields more satellite cells. This method was established by Bischoff (1974) using rat muscle and subsequently adapted for farm animals, such as sheep (Dodson et al. 1986), chicken (Yablonka-Reuveni et al. 1987), cattle (Dodson et al. 1987), turkeys (McFarland et al. 1988), fish (Powell et al. 1989), pigs (Doumit and Merkel 1992) and horses (Greene and Raub 1992). The myofiber isolation method is advantageous if it is necessary to maintain the satellite cells in their characteristic position (niche) and in a quiescent state. This method was pioneered by Bekoff and Betz (1977) and Bischoff (1986) using rat skeletal muscle and was later performed with goat and pig muscle fibers (Yamanouchi et al. 2009, Wilschut et al. 2010).

Primary cell cultures of satellite cells derived from skeletal muscle tissue can be performed from one individual animal or as a cell pool consisting of cells from several animals. Cell pooling in itself was and still is a matter of discussion (Stoddart et al. 2012). However, it is generally agreed that this technique offers an opportunity to perform long-term projects with a broad spectrum of experiments, analyses and multiple replications. In addition, it is known that pooling of cells from multiple donors reduces the biological variability of the different cell donors. Working with cell pools therefore requires a clear and transparent description of their establishment and composition.

In the present study, we used real-time impedimetric cell monitoring to compare the growth profile of three different cell pools of porcine proliferating muscle cells with that

of corresponding unpooled cells of several donor pigs. Our objective was to determine whether cell pool growth is similar to the unpooled cells from individual donors.

Isolation of satellite cells

All procedures were in accordance with the German Law of Animal Protection. In this study, we used skeletal muscle tissue from 26 piglets with normal birth weight (1.36 ± 0.15 kg) at three different ages (Pool 1 – *M. longissimus*, n = 6, day 4 of age, male; Pool 2 – *M. rhomboideus*, n = 10, day 5 of age, female; Pool 3 – *M. rhomboideus*, n = 10, day 20 of age, female). These piglets were from two different research projects (project 1 = Pool 1; project 2 = Pool 2 and 3) that were carried out at the pig breeding facility of the Leibniz Institute for Farm Animal Biology (FBN, Dummerstorf, Germany). Piglets were killed at the FBN slaughterhouse using exsanguination after captive-bolt pistol (4 and 5 days of age) or electro stunning (20 days of age). The skeletal muscle tissue was dissected and washed in enriched phosphate buffered saline (PBS-D; 144 mM NaCl, 5.4 mM KCl, 25 mM glucose, 14 mM sucrose, 5 mM Na_2HPO_4, 50 IU/mL penicillin, 50 µg/mL streptomycin and 1 µg/mL phenol red, adjusted to pH 7.4 at 22°C) until the isolation procedure (Fig. 1). The muscle samples (Pool 1: 14.84 ± 0.79 g from *M. longissimus*; Pool 2: 4.24 ± 0.79 g = the whole *M. rhomboideus*, Pool 3: 6.23 ± 1.11 g = the whole *M. rhomboideus*) were trimmed of visible connective tissue, weighed, washed with PBS-D and minced with scissors. The isolation procedure has been described by Mau et al. 2008b, but we have modified the enzymatic digestions and the Percoll gradient centrifugation steps (**Fig. 2.1**). Briefly, the cells were dissociated by fractional digestion using a mixture of 0.2% collagenase (Collagenase type I, CLS I, C1-22, Biochrom, Berlin, Germany), 0.01% DNase (DNase I, AppliChem, Darmstadt, Germany) and 0.025% trypsin (Sigma-Aldrich, Taufkirchen, Germany) in Hank's balanced salt solution (HBSS; Biochrom) for 20 min at 37°C with medium stirring speed. The digestion was then stopped by being placed on ice for 2 min. The supernatant was removed, diluted 1:1 with isolation medium (MEMα (GIBCO Thermo Fisher, Schwerte, Germany) supplemented with 0.2 M L-glutamine (Carl Roth, Karlsruhe; Germany), 100 IU/mL penicillin (Biochrom), 100 µg/mL streptomycin (Biochrom), 2.5 µg/mL amphotericin (Sigma-Aldrich) and 10% fetal bovine serum (FBS; Sigma-Aldrich)), sifted through a 100-µm nylon strainer (Corning, Wiesbaden, Germany) and centrifuged for 10 min at

800 × g and 4°C. The supernatant was then discarded, and the pellet was suspended in 5 mL of isolation medium. The remaining digestion solution was replenished with 25 mL of the abovementioned enzyme mixture in HBSS. The procedure was repeated twice. For each animal, the cell suspensions obtained after each digestion were pooled and sifted through a 70-µm nylon strainer. The satellite cells were enriched by Percoll (Sigma-Aldrich, 20% and 60% in PBS) gradient centrifugation. Specifically, 1.5 mL of a 60% Percoll solution was added to a 15-mL Falcon tube, and 11.5 mL of a 20% Percoll solution was then layered on top of the first one. The gradient was finally completed by adding 2 mL of the cell suspension. The gradients were centrifuged at 15,000 × g for 9 min at 4°C with the brakes off. The layer containing the satellite cells (at the interface of the 20% and 60% Percoll solutions, see Fig. 1) was carefully removed, transferred to isolation medium and centrifuged at 700 × g for 10 min at 4°C. The supernatants were discarded, and the cell pellets were washed twice with isolation medium. Finally, the cell pellet was resuspended, the number of viable cells was determined (Countess, Invitrogen), and approximately 10^5 cells/cm² were seeded on gelatin-coated (0.1%) 100-mm plastic cell culture dishes (Sarstedt, Sarstedt, Germany) in 15 mL growth medium (DMEM (Biochrom) supplemented with 0.2 M L-glutamine (Carl Roth), 100 IU/mL penicillin (Biochrom), 100 µg/mL streptomycin (Biochrom), 2.5 µg/mL amphotericin (Sigma-Aldrich), 10% FBS (Sigma-Aldrich) and 10% donor horse serum (HS; Sigma-Aldrich)). After 48 h incubation (37°C, 6% CO_2), the cells were washed with PBS (Biochrom), and the medium was changed. After 72 h, the cell monolayer was approximately 90 to 95% confluent. These cells were then harvested and cryopreserved as described by Mau et al. 2008.

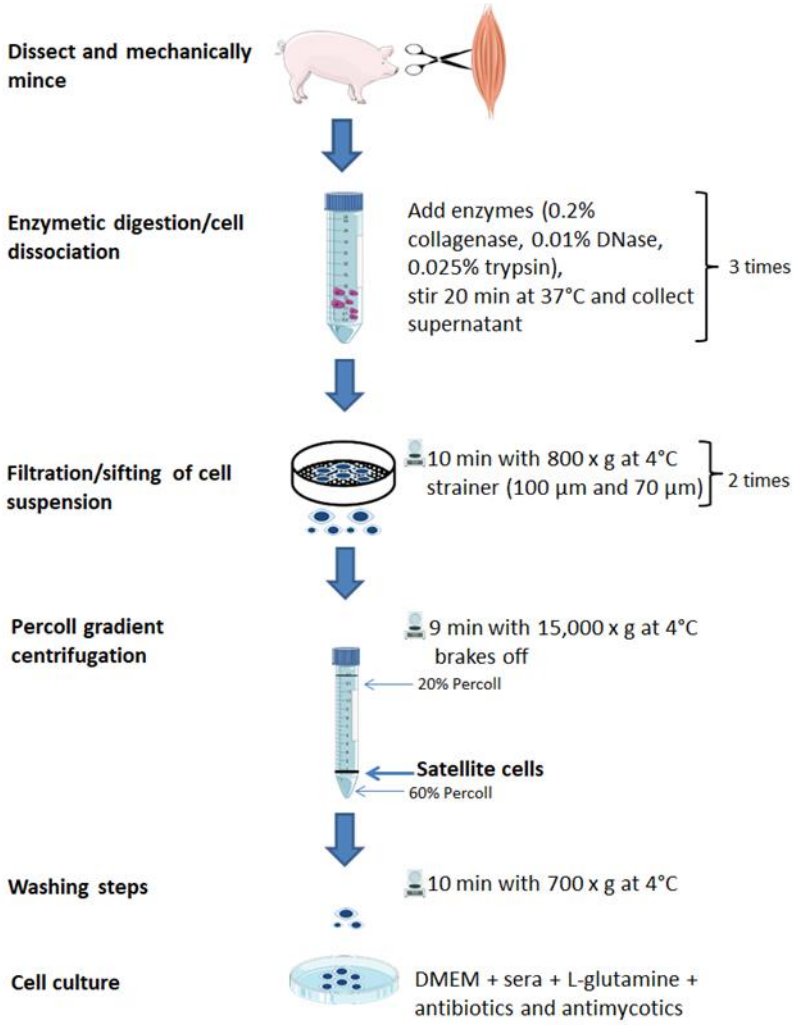

Figure 2.1: Schematic overview of the satellite cell isolation procedure using porcine skeletal muscle tissue. This procedure is adapted from Mau et al. 2008.

Establishment of cell pools

For the establishment of cell pools, approximately 3×10^6 satellite cell progenies of each animal were pooled. For Pool 1, the cells were grown for 24 h (approximately 90% confluent). Pools 2 and 3 were established with the aim of obtaining the largest possible cell pools. Therefore, the cells were grown until reaching 90% confluence and then split in a ratio of 1:3 and harvested upon reaching a confluence of 90%. Cells were aliquoted (2×10^6 cells per vial) and cryopreserved at passage number two for Pool 1 and three for Pools 2 and 3.

For each cell pool, a cell aliquot (1×10^6 cells) was taken and seeded on a gelatin-coated 100-mm cell culture dish (Sarstedt) for the estimation of the percentage of myogenic cells by immunostaining using an antibody against desmin (D1033 mouse monoclonal anti-desmin antibody, Sigma-Aldrich), which is characteristic of replicating myoblasts (Kaufman and Foster 1988) and 4,6-diami-dino-2-phenylindole (DAPI, Carl Roth) for the nuclei. After approximately 24 h, the cells were harvested upon reaching a confluence of 80% (**Fig. 2.2 A, B**). Cell fixation and immunostaining were performed according to Mau et al. (2008) and analyzed using the Q-Win imaging software (Leica, Wetzlar, Germany) on at least 8000 cells per pool. In the two representative pictures (**Fig. 2.2 C, D**), desmin positive cells are in green and the nuclei in red (Pool 1: 97 ± 1%; Pool 2: 98 ± 1%; Pool 3: 95 ± 2%).

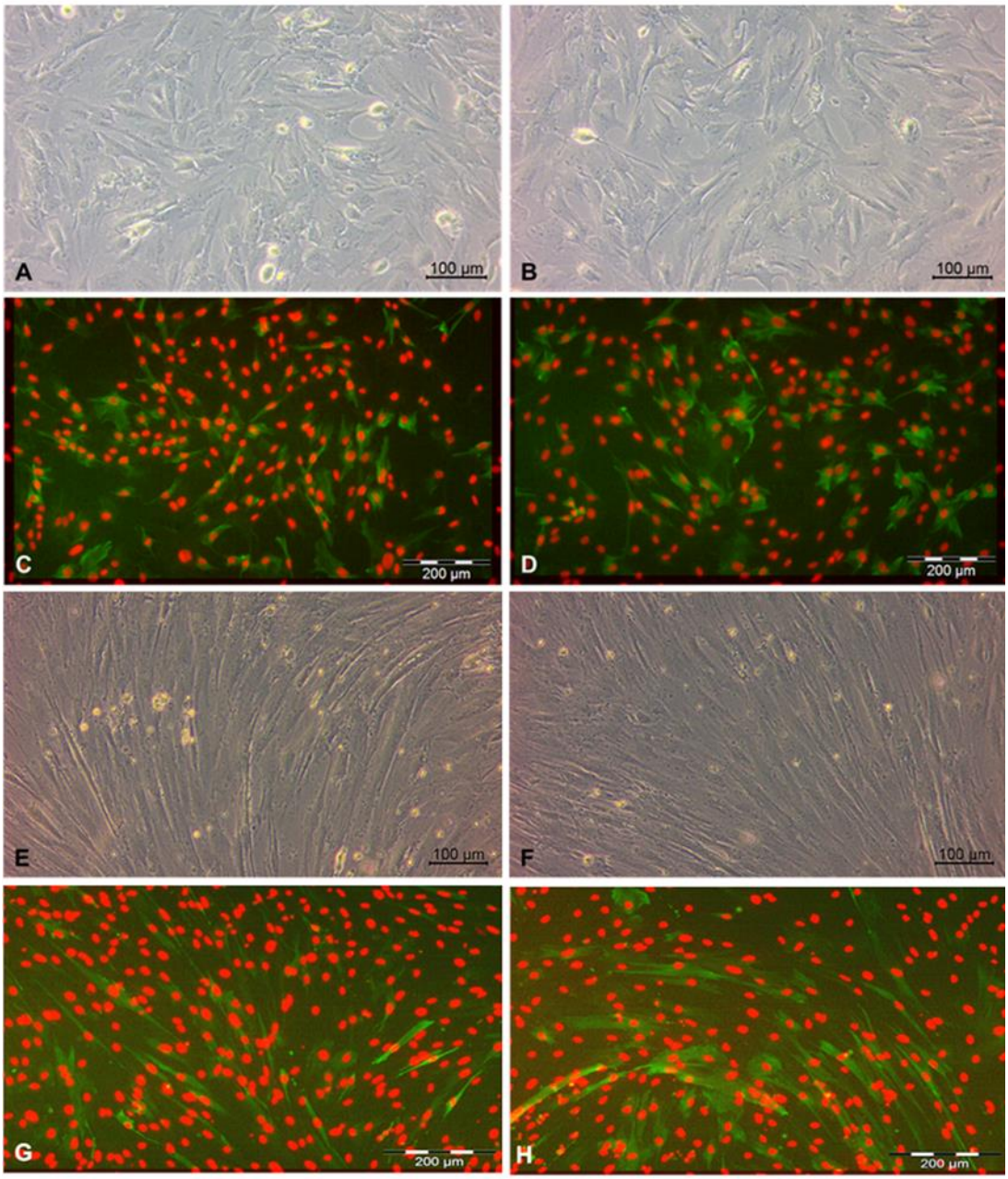

Figure 2.2: Cell pools derived from satellite cells of M. rhomboideus at 5 (A, C, E, G) or 20 days (B, D, F, H) of age. Myoblasts were seeded on gelatin-coated dishes and grow for 24 h (A, B). Desmin-positive cells (C, D, in green) were determined by immunostaining using a D1033 mouse monoclonal anti-desmin antibody. The stained nuclei appeared red (DAPI). Pool 2 (C) and Pool 3 (D) exhibited 98 ± 1 % and 95 ± 2 % desmin positive cells, respectively (> 8000 cells per pool were analyzed). Myoblasts were seeded on Geltrex™ (growth factor reduced, 1:100)-coated dishes and allowed to grow and differentiate for 11 days (E, F). Pool 2 (G) and Pool 3 (H) exhibited 53 ± 1 % and 55 ± 1 % differentiating myotubes. A myotube was defined as a desmin-positive cell containing three or more nuclei (DAPI). Ten representative pictures from each cell pool or corresponding unpooled cells were analysed (Q-Win imaging system, Leica).

Myogenic phenotype of pooled vs. unpooled cells

The myogenic phenotype were determined by the mRNA expression of the satellite cell marker *PAX7 (*paired box transcription factor 7) and the muscle-specific transcription factors *MYOD* (myogenic differentiation factor) and *MYOG* (myogenin) after 72 h of proliferation. Therefore, for each cell pool and their corresponding unpooled cells, a cell aliquot (1×10^6 cells) was taken and seeded on a gelatin-coated 100-mm cell culture dish (Sarstedt) with growth medium for 72 h. The RNA isolation (Kalbe et al. 2008), the reverse transcription and real-time PCR procedures, including primer information (Kalbe et al. 2018), were previously described. Data are expressed as arbitrary units after normalisation with the endogenous reference gene *HPRT1* (hypoxanthine phosphoribosyltransferase 1). There were no differences between the cell pools and their corresponding unpooled cells with regard to the mRNA expression of *PAX7* (Pool 1: 0.034 ± 0.011 vs. 0.025 ± 0.004, P = 0.48; Pool 2: 0.005 ± 0.004 vs. 0.003 ± 0.001; P = 0.59; Pool 3: 0.003 ± 0.002 vs. 0.004 ± 0.001, P = 0.86). Moreover, the mRNA expression of *MYOD* (Pool 1: 0.100 ± 0.072 vs. 0.130 ± 0.030, P = 0.72; Pool 2: 0.011 ± 0.007 vs. 0.005 ± 0.002; P = 0.41; Pool 3: 0.004 ± 0.005 vs. 0.005 ± 0.002, P = 0.93) and *MYOG* (Pool 1: 0.989 ± 0.333 vs. 0.881 ± 0.136, P = 0.78; Pool 2: 0.053 ± 0.020 vs. 0.016 ± 0.007; P = 0.13; Pool 3: 0.024 ± 0.012 vs. 0.010 ± 0.004, P = 0.30) did not differ between cell pool and their corresponding unpooled cells.

To estimate the differentiation capacity, 4×10^5 cells per cell pool or unpooled cells were seeded in Geltrex™ (growth factor reduced, 1:100, Gibco Thermo Fisher)-coated 100-mm cell culture dishes. Cells were grown in growth medium for 4 days, in growth medium 2 (DMEM (Biochrom) supplemented with 0.2 M L-glutamine (Carl Roth), 100 IU/mL penicillin (Biochrom), 100 µg/mL streptomycin (Biochrom), 2.5 µg/mL amphotericin (Sigma-Aldrich), 10% FBS (Sigma-Aldrich) and 1 µM insulin (Sigma-Aldrich)) for 1 day and then in serum-free differentiation medium (MEM (Biochrom) supplemented with 0.2 M L-glutamine (Carl Roth), 100 IU/mL penicillin (Biochrom), 100 µg/mL streptomycin (Biochrom), 2.5 µg/mL amphotericin (Sigma-Aldrich), 1 µM insulin (Sigma-Aldrich), 1 µM cytosine β-D-arabinofuranoside (Sigma-Aldrich), 0.5 mg/mL bovine serum albumin (Sigma-Aldrich), 0.1 nM dexamethasone (Sigma-Aldrich), 0.5 µg/mL linoleic acid (Sigma-Aldrich) and 100 µg/mL transferrin (bovine holoform, Sigma-Aldrich)) for 6 days. The estimation of fusion degree was performed

after 6 days of differentiation as described by Mau et al. 2008 (**Fig. 2.2 E-H**). A myotube was defined as three or more nuclei in a cell membrane. There were no significant differences in the fusion degree between of each cell pool (1, 2 and 3) and their corresponding unpooled cells (Pool 1: 32.89 ± 1.69% vs. 29.16 ± 4.13%, P = 0.44; Pool 2: 52.74 ± 1.20% vs. 50.04 ± 3.59%, P = 0.50; Pool 3: 54.91 ± 1.14% vs. 51.34 ± 3.60%, P = 0.37). The observed fusion degrees are in agreement with other porcine studies (Doumit and Merkel 1992, Baquero-Perez et al. 2012).

Comparison of pooled vs. unpooled cells

Real-time myoblast proliferation was monitored by recording the impedance every 30 min. This monitoring was carried out with the xCELLigence RTCA SP system, using 96-well culture plates with electrodes in the bottom of each well (e-plate 96, ACEA Biosciences Inc., San Diego, USA). Data are presented as the cell index (arbitrary units), which corresponds to the changes in impedance over a specific time period, in our case, a 72-h growing period. Impedance as a cellular readout was previously established (Giaever and Keese 1993) and previously used for primary skeletal muscle myoblasts (Sente et al. 2016). Most publications using impedance-based label-free technology have focused on the effects of various compounds on cellular adhesion and proliferation (Atienzar et al. 2011, Will et al. 2012). For all experiments, the cells were thawed rapidly in a water bath at 37°C and washed with growth medium. The e-plates were coated with gelatin, and 5000 (Pool 1 – *M. longissimus*) or 4000 (Pool 2 and 3 – *M. rhomboideus*) cells were seeded per well using growth medium. For experiments with unpooled cells from individual animals of Pool 1, the cells were seeded in two wells (duplicate), and cell pool 1 was present in four wells. The experiment was repeated 6 times with varying plate positions. After 24 h, half of the culture medium was changed, and a complete medium change was performed after 48 h. For Pool 2 and Pool 3, three different xCELLigence runs were performed. For each run, three wells were seeded with unpooled cells from each animal and 10 wells per pool. The medium was changed after 48 h. During all xCELLigence runs, 14 individual wells were excluded because of erroneous values. The cell indices profile (given as mean ± standard deviation) over the 72-h growth period is shown in **Figure 2.3**. For each of the three cell pools, the cell index profiles of pooled cells were similar to those of the unpooled cells from the corresponding pig donors.

Therefore, these results suggest that the use of pooled cells is an appropriate method to reflect the average proliferative growth behavior of unpooled cells.

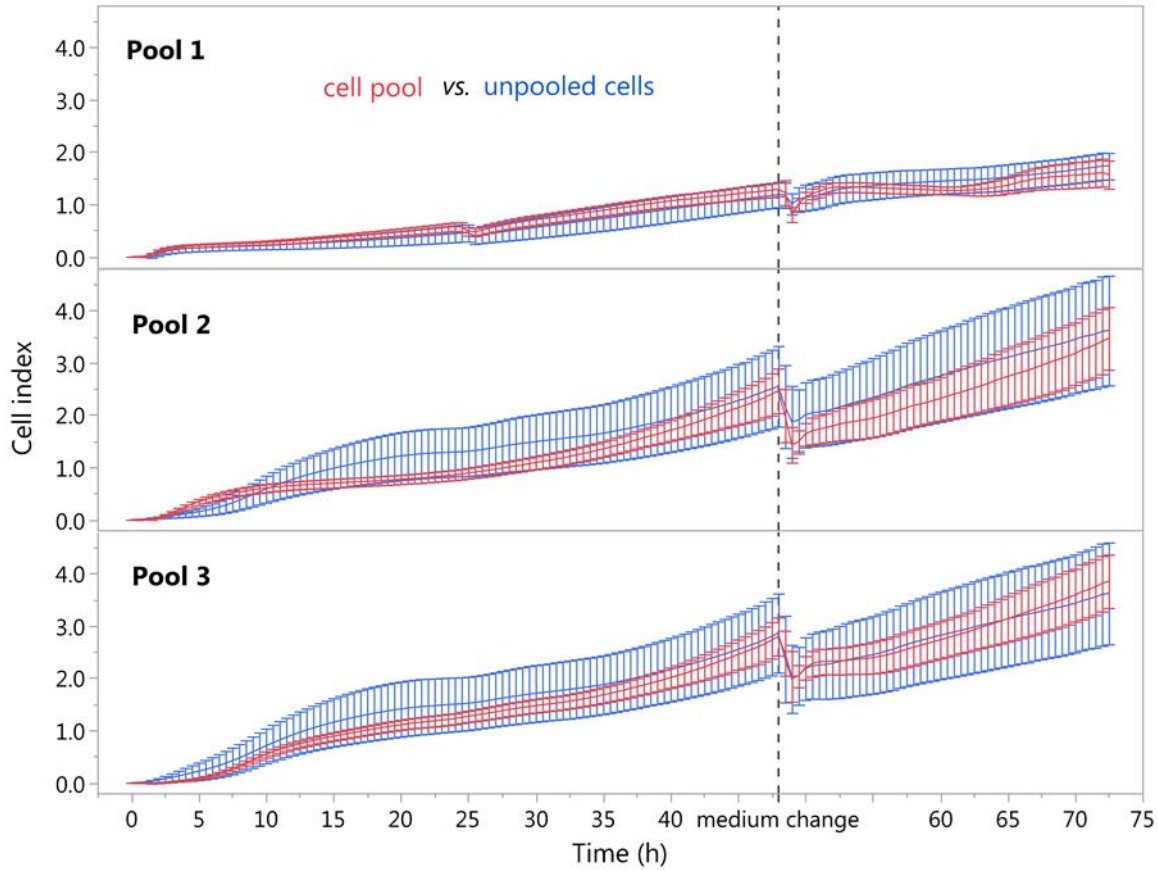

Figure 2.3: Cell index (means ± standard deviation) for three different cell pools (Pool 1-3) and their corresponding unpooled cells was measured in real time every 30 min over 72 h using the xCELLigence system (ACEA Biosciences Inc). The cell index is a dimensionless value that measures the relative change in electrical impedance to represent the cell status. The cell pools are shown in red and represent the whole unpooled cells from the corresponding animals shown in blue.

For statistical analysis, data were subjected to analysis of variance using the mixed procedure in SAS (version 9.2, SAS Inst Inc., Cary, NC). Samples (cell pool or unpooled cells) and the replication of the experiment (six for Pool 1 and three for Pool 2 and 3) were used as fixed factors. Differences between the least squares means were tested with Tukey tests. There were no significant differences between the average cell index values of each cell pool (1, 2 and 3) and their corresponding unpooled cells (Pool 1: 0.855 ± 0.150 vs. 0.818 ± 0.061, P = 0.83; Pool 2: 1.484 ± 0.386 vs. 1.706 ± 0.129, P = 0.60; Pool

3: 1.766 ± 0.357 vs. 1.929 ± 0.119, P = 0.68). In agreement with these findings, the cell indices of the cell pools and their corresponding unpooled cells were similar in each experimental replication (Pool 1: P = 1.00; Pool 2: P ≥ 0.76, Pool 3: P ≥ 0.98, data not shown). The slope (1/h) describes the steepness and incline of the cell index curve and is an indication of the growth rate. In this study, the slope was calculated over the experimental period of 72 h. The averaged slope values were unchanged between the unpooled cells of the animals and the corresponding cell pools (**Table 2.1**, Pool 1: P = 0.34, Pool 2: P = 0.73 and Pool 3: P = 0.46).

Table 2.1: Comparison of slopes over a growing period of 72 hours for pooled porcine myoblasts and their corresponding unpooled cells

Pool	**Donor tissue**	**Experimental replication**	**Slope (1/hour)[a]:**	
			Cell pool	**Unpooled cells**
Pool 1	*M. long.*, 4 d	1	0.024 ± 0.003	0.026 ± 0.001
1.00				
		2	0.024 ± 0.003	0.025 ± 0.001
1.00				
		3	0.019 ± 0.004	0.025 ± 0.002
0.94				
		4	0.022 ± 0.003	0.025 ± 0.001
0.99				
		5	0.021 ± 0.003	0.023 ± 0.001
1.00				
		6	0.020 ± 0.004	0.025 ± 0.002
0.99				
		average	0.022 ± 0.003	0.025 ± 0.001
0.34				
Pool 2	*M. rhom.*, 5 d	1	0.038 ± 0.012	0.034 ± 0.004
1.00				
		2	0.034 ± 0.010	0.042 ± 0.004
0.97				
		3	0.051 ± 0.009	0.056 ± 0.003
1.00				
		average	0.041 ± 0.008	0.044 ± 0.003
0.73				
Pool 3	*M. rhom.*, 20 d	1	0.044 ± 0.007	0.038 ± 0.003
0.94				
		2	0.045 ± 0.007	0.041 ± 0.003
0.99				
		3	0.056 ± 0.005	0.054 ± 0.002
1.00				
		average	0.048 ± 0.005	0.044 ± 0.002
0.46				

[a] The slope was calculated with the xCELLigence (ACEA Biosciences Inc) software (RTCA, Version 1.2.1) using the following formula: Cell index = slope * time + intercept and is presented as least squares means ± standard errors.
M. long. – *M. longissimus*, *M. rhom.* – *M. rhomboideus*, P – P value of Tukey test

Moreover, for each experimental replication, there was no difference in slope values between the unpooled cells of the animals and their corresponding pools (Table 1, $P \geq 0.94$). The doubling time (h) is also an indicator for proliferative potential of the cells describing the period of time required for the cell index to double. Doubling time was calculated over a 67 h period (from 5 h to 72 h), starting at 5 h to allow the myoblasts to

attach after seeding. The average doubling times were also unchanged between the unpooled cells and their corresponding cell pools (**Table 2.2**, Pool 1: P = 0.09, Pool 2: P = 0.88 and Pool 3: P = 0.36).

Table 2.2: Comparison of doubling time over a growing period from 5 to 72 hours for pooled porcine myoblasts and their corresponding unpooled cells

Pool	**Donor tissue**	**Experimental replication**	**Doubling time (hour)[a]: Cell pool**	**Unpooled cells**	**P**
Pool 1	*M. long.*, 4 d	1	24.00 ± 2.82	19.36 ± 1.20	0.92
		2	22.68 ± 3.18	20.48 ± 1.36	1.00
		3	23.22 ± 2.27	19.29 ± 0.97	0.90
		4	24.78 ± 3.31	20.39 ± 1.41	0.98
		5	25.75 ± 2.33	19.23 ± 0.99	0.34
		6	24.66 ± 2.96	18.07 ± 1.26	0.66
		average	24.18 ± 2.02	19.47 ± 0.86	0.09
Pool 2	*M. rhom.*, 5 d	1	25.19 ± 4.62	22.70 ± 1.64	1.00
		2	25.26 ± 4.53	25.82 ± 1.54	1.00
		3	22.89 ± 3.53	23.24 ± 1.12	1.00
		average	24.45 ± 3.35	23.92 ± 1.15	0.88
Pool 3	*M. rhom.*, 20 d	1	19.77 ± 2.64	24.30 ± 1.53	0.68
		2	24.86 ± 4.44	28.70 ± 2.22	0.97
		3	25.48 ± 3.86	25.52 ± 1.93	1.00
		average	23.37 ± 2.55	26.18 ± 1.30	0.36

[a] The doubling time was calculated with the xCELLigence (ACEA Biosciences Inc) software (RTCA, Version 1.2.1) using the following formula: Cell index = A* 2^(t/CI doubling-time) and is presented as least squares means ± standard errors.
M. long. – *M. longissimus*, *M. rhom.* – *M. rhomboideus*, P – P value of Tukey test

In addition, for each experimental replication, there was no difference in doubling time between the unpooled cells and their corresponding pools (Table 2, $P \geq 0.34$). The current study results clearly show that it would be appropriate to use the three different cell pools in different experimental setups because they perfectly reflected their corresponding six (Pool 1) or ten (Pool 2 and 3) donor piglets based on the real-time monitoring of growth behavior. To ensure that all donors respond to the same extent within their individual variability, we therefore recommend carefully establishing representative muscle cell pools derived from satellite cells of muscle tissue from several donors.

In conclusion, the following requirements are indispensable when using cell pools derived from several donors: 1) a detailed data record for the donor's background including the number of animals and their gender, the donor tissue (specific muscle) and birth weight, which is known to strongly affect myogenesis (Paredes et al. 2013); 2) a detailed description of the isolation and establishment procedures for the cell pools. It is also important to mention that the seeded cell number and the developmental stage (cell passage number) for each donor animal should always be equal; 3) a minimum of three experimental replicates is needed to minimize variations.

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CHAPTER 3

Based on

Effects of temperature on proliferation of myoblasts from donor piglets with different thermoregulatory maturities

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Effects of temperature on proliferation of myoblasts from donor piglets with different thermoregulatory maturities

Abstract

Background: Climate change and the associated risk for the occurrence of extreme temperature events or permanent changes in ambient temperature are important in the husbandry of farm animals. The aim of our study was to investigate the effects of permanent cultivation temperatures below (35°C) and above (39°C, 41°C) the standard cultivation temperature (37°C) on porcine muscle development. Therefore, we used our porcine primary muscle cell culture derived from satellite cells as an in vitro model. Neonatal piglets have limited thermoregulatory stability, and several days after birth are required to maintain their body temperature. To consider this developmental step, we used myoblasts originating from thermolabile (five days of age) and thermostable piglets (twenty days of age).

Results: The efficiency of myoblast proliferation using real-time monitoring via electrical impedance was comparable at all temperatures with no difference in the cell index, slope or doubling time. Both temperatures of 37°C and 39°C led to similar biochemical growth properties and cell viability. Only differences in the mRNA expression of myogenesisassociated genes were found at 39°C compared to 37°C with less MYF5, MYOD and MSTN and more MYH3 mRNA. Myoblasts grown at 35°C are smaller, exhibit higher DNA synthesis and express higher amounts of the satellite cell marker PAX7, muscle growth inhibitor MSTN and metabolic coactivator PPARGC1A. Only permanent cultivation at 41°C resulted in higher HSP expression at the mRNA and protein levels. Interactions between the temperature and donor age showed that MYOD, MYOG, MYH3 and SMPX mRNAs were temperature-dependently expressed in myoblasts of thermolabile but not thermostable piglets.

Conclusions: We conclude that 37°C to 39°C is the best physiological temperature range for adequate porcine myoblast development. Corresponding to the body temperatures of

piglets, it is therefore possible to culture primary muscle cells at 39°C. Only the highest temperature of 41°C acts as a thermal stressor for myoblasts with increased HSP expression, but it also accelerates myogenic development. Cultivation at 35°C, however, leads to less differentiated myoblasts with distinct thermogenetic activity. The adaptive behavior of derived primary muscle cells to different cultivation temperatures seems to be determined by the thermoregulatory stability of the donor piglets.

Keywords

Satellite cells, myoblasts, temperature, thermal stress, age of donor, pig

Background

Climate change has caused an associated risk for the occurrence of extreme heat events in farm animals that have been subjected to heat stress (Horton et al. 2016). In conventional pig husbandry, heat abatement plays a major role (St-Pierre et al. 2003), whereas the challenges in free-range farming are quite different. During birth, piglets must overcome many challenges, such as respiration, digestion, nutrition and thermoregulation, having to regulate their own body temperature to survive (Herpin et al. 2002a). Adaptive thermogenesis is a specialized type of heat production and occurs in brown adipose tissue and skeletal muscle (Fuller-Jackson & Henry 2018). Newborn piglets do not possess brown adipose tissue (Trayhurn et al. 1989, Herpin et al. 2002b); for this reason, they are thermolabile after birth, and an appropriate thermal environment is needed (Tuchscherer et al. 2000). Body temperature rises to the physiological value of 39 °C within 48 h p.p. (Mount 1968, Berthon et al. 1993), and after the first week of age, thermoregulatory functions are fully developed (Curtis & Rogler 1970). Satellite cells are quiescent myogenic stem cells (Mauro 1961). They are involved in hypertrophic muscle growth and regeneration and maintain the muscle stem cell reservoir. The first isolation was performed by using rat muscle (Bischoff 1974). In this in vitro model, satellite cells develop into proliferating and differentiating progenies and are therefore a suitable model for muscle biology research. This approach provides the opportunity to directly

investigate the influence of cultivation conditions such as changes in temperature. For instance, the effects of different but permanent cultivation temperatures on primary human skeletal muscle cells (range of 37 to 41 °C) and on C2C12 cells (an immortalized mouse muscle cell line, range of 35 to 41 °C) were investigated (Yamaguchi et l. 2010, Guo et al. 2016, Sajjanar et al. 2019). For avian primary muscle cell cultures, the range for permanent temperature experiments was from 33 to 43 °C (turkey (Clark et al. 2016, Clark et al. 2017, Reed et al. 2017a, Reed et al. 2017b, chicken (Harding et al. 2015, Harding et al. 2016)). In pigs, the effects of heat stress during housing are already well investigated (Le Bellego et al. 2002, Patience et al. 2005, Hao et al. 2014), whereas in vitro studies in muscle cell cultures are still rare. The first studies with isolated satellite cells of pigs (*M. semitendinosus* or *M. longissimus*) focused on precultivation at a control temperature and subsequent heat stress at 40.5 °C or 41 °C (Kamanga-Sollo et al.2011, Gao et al. 2015). The aim of our study was to investigate the effect of permanent cultivation temperatures below (35 °C) and above (39 °C and, 41 °C) the standard cultivation temperature (37 °C) on the proliferative growth of satellite cell progenies originating from *M. rhomboideus* of thermolabile (five days of age) and thermostable piglets (twenty days of age). We used real-time impedimetric cell growth monitoring, morphological and biochemical properties and the expression analysis of myogenesisassociated genes to characterize the temperaturedependent effects while considering the thermoregulatory maturity of the donor piglets.

Results

Real-time growth monitoring

Proliferative growth at 35°, 37°, 39° and 41 °C was monitored in real time with the xCELLigence RTCA SP system (**Fig. 3.1**) over a period of 72 h. The cell index (CI) was used to measure the relative change in the electrical impedance that represents the cell status. Important for the impedance are the number and the size of cells that are attached on the electrode in the bottom of the eplate. The average CI was unaffected by the

temperature (**Table 3.1**, *P* = 0.905) or pool (*P* = 0.696), with no interaction between the temperature and pool (*P* = 0.978). The doubling time (h) describes the period required to double a CI value. The doubling time was calculated over a 67 h period (from 5 to 72 h), starting at 5 h to allow the myoblasts to attach after seeding. The average doubling time was unaffected by the temperature (**Table 3.1**, *P* = 0.524). However, the average doubling time was affected by the pool, with a higher doubling time of pool 5 (P = 0.007), but not by the interaction of the temperature and pool (*P* = 0.934). The slope (1/h) characterizes the steepness, inclination or change of a curve. The slope was not affected by the temperature (**Table 3.1**, *P* = 0.323), pool (*P* = 0.297) or their interaction (*P* = 0.385).

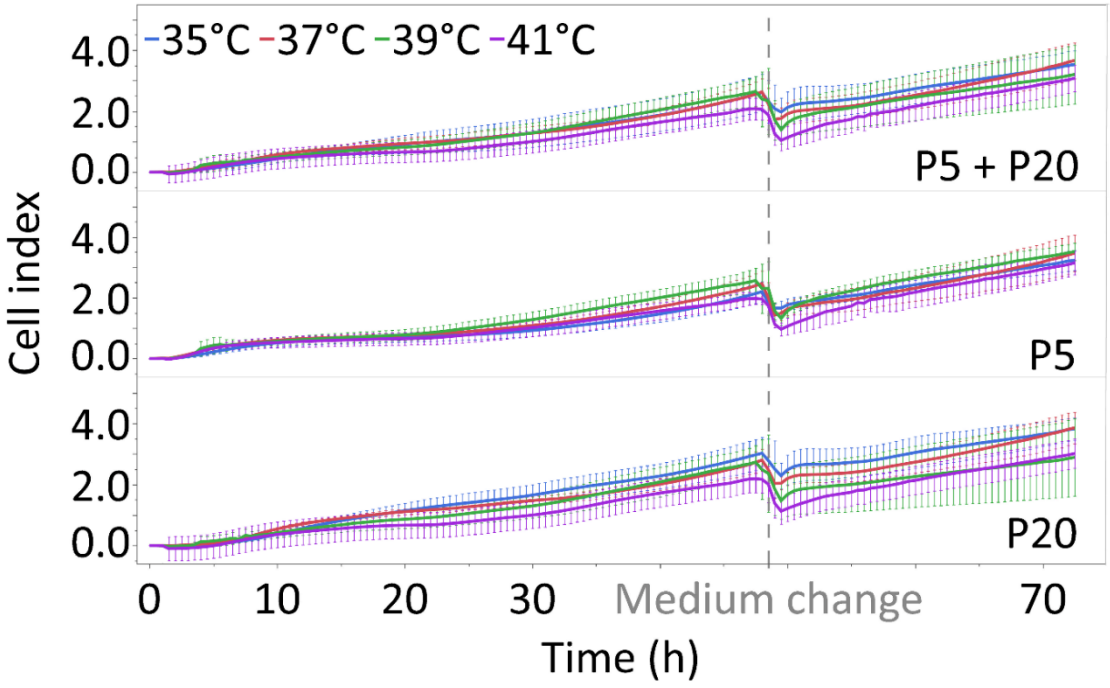

Figure 3.1: Cell indices (means ± standard deviations) measured in real time every 30 min over 72 h using the xCELLigence RTCA SP system in all myoblasts (P5 + P20), pool 5 (P5) and pool 20 (P20) that were permanently cultured at 35°, 37°, 39° or 41 °C. Values were generated from three independent experiments.

Table 3.1: Average cell index parameters (least square means ± standard errors) generated from real-time monitoring of 72 h proliferative growth.

Parameter	**Temperature (T)**				**Pool (P)**		**T**	**P**	**T×P**
	35°C	37°C	39°C	41°C	5	20	***P***	***P***	***P***
Cell index (arbitrary units)	1.67 ± 0.12	1.63 ± 0.12	1.58 ± 0.12	1.37 ± 0.12	1.47 ± 0.09	1.65 ± 0.09	0.905	0.696	0.978
Slope (1/h)	0.048 ± 0.004	0.045 ± 0.004	0.042 ± 0.004	0.039 ± 0.004	0.041 ± 0.002	0.045 ± 0.002	0.323	0.297	0.385
Doubling time[1] (h)	20.4 ± 1.2	22.7 ± 1.3	20.6 ± 1.3	21.8 ± 0.9	23.3 ± 0.9	19.4 ± 0.9	0.524	0.007	0.934

[1] - The doubling time was calculated over a 67 h period (from 5 to 72 h), starting at 5 h to allow the myoblasts to attach after seeding.

Biochemical properties of growth

In a combined assay, the DNA and protein contents (**Table 3.2**) were detected in the monolayers. The DNA content (µg/well) was equivalent to the cell number and was affected by the temperature ($P = 0.006$) but not by the pool ($P = 0.606$) or the interaction of both ($P = 0.930$). Higher contents were found at 35 °C and 39 °C than at 37 °C ($P \leq 0.034$), with an unchained content at 41 °C ($P \geq 0.317$). The protein content (µg/well) was unchanged between the temperatures ($P = 0.894$), pool ($P = 0.785$) or their interaction ($P = 0.869$).

The cell proliferation ELISA is based on the measurement of 5-bromo-2′-deoxyuridine (BrdU) incorporation during DNA synthesis (**Table 3.2**). DNA synthesis was affected by the temperature ($P < 0.001$), with a higher rate at 35 °C than at 37 °C and 41 °C ($P \leq 0.013$) but not by the pool ($P = 0.431$) or the interaction of both ($P = 0.794$).

Proliferating cell nuclear antigen (PCNA, **Table 3.3**) is a proliferation marker, and mRNA expression was affected by the temperature ($P < 0.001$) but not by the pool ($P = 0.060$) or the interaction of both ($P = 0.174$). The highest mRNA expression was found at 35 °C and decreased with increasing temperature. In addition, the mRNA expression at 37 °C was increased compared to 39 and 41 °C ($P < 0.001$).

Apoptosis was investigated using a commercial TUNEL assay (**Table 3.2**), and the mRNA expression of the gene encoding the defender against apoptotic cell death (DAD1, Table 3). There were not temperaturedependent effects on the percentage of TUNEL-positive cells and mRNA expression of DAD1 ($P \geq 0.141$), pooldependent effects ($P \geq 0.253$) or the interaction between the temperature and pool on both apoptotic properties ($P \geq 0.415$).

Lactate dehydrogenase (LDH, **Table 3.2**) is an enzyme that is ubiquitously present in all cells and will be liberated from the cell interior in the cell culture supernatant after cell damage. Significant effects of the temperature ($P < 0.001$) and pool ($P = 0.032$) were detected, but no interaction was detected between the two ($P = 0.715$). Increased LDH activity was found at 35 °C compared to all temperatures ($P \leq 0.002$) and in pool 20 compared to pool 5 ($P = 0.032$).

Table 3.2: Biochemical properties (least square means ± standard errors) of growth after 72 h of proliferation.

Parameter	Temperature (T)				Pool (P)		T	P	T × P
	35°C	37°C	39°C	41°C	5	20	*P*	*P*	*P*
DNA content (µg/well)	0.47 ± 0.04 [a]	0.28 ± 0.04 [b]	0.54 ± 0.04 [a]	0.43 ± 0.04 [ab]	0.44 ± 0.03	0.42 ± 0.03	0.006	0.606	0.930
Protein content (µg/well)	20.62 ± 3.34	22.57 ± 3.34	21.35 ± 3.34	24.04 ± 3.34	21.68 ± 2.36	22.61 ± 2.36	0.894	0.785	0.869
DNA synthesis (Abs 450nm)	1.62 ± 0.07 [a]	1.27 ± 0.07 [b]	1.34 ± 0.07 [ab]	1.11 ± 0.07 [b]	1.31 ± 0.05	1.36 ± 0.05	< 0.001	0.431	0.794
LDH activity (IU/L)	41.85 ± 2.27 [a]	24.92 ± 2.27 [b]	22.39 ± 2.27 [b]	26.85 ± 2.27 [b]	26.30 ± 1.61	31.70 ± 1.61	< 0.001	0.032	0.715
TUNEL$^+$ cells (%)	0.05 ± 0.01	0.02 ± 0.01	0.04 ± 0.01	0.07 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.141	0.681	0.712

Abs - absorbance, LDH - lactate dehydrogenase

[a, b] Labeled least square means within a row with different letters differ ($P < 0.05$).

Table 3.3: Expression of genes associated with cellular development and stress (least square means ± standard errors) after 72 h of proliferation.

Gene	Temperature (T)				Pool (P)		T	P	T × P
	35°C	37°C	39°C	41°C	5	20	*P*	*P*	*P*
PCNA mRNA	1.66 ± 0.05 [a]	0.93 ± 0.05 [b]	0.55 ± 0.05 [c]	0.49 ± 0.05 [c]	0.85 ± 0.04	0.96 ± 0.04	< 0.001	0.060	0.174
DAD1 mRNA	0.87 ± 0.05	0.78 ± 0.05	0.85 ± 0.05	0.96 ± 0.05	0.83 ± 0.04	0.89 ± 0.04	0.159	0.253	0.415
PPARGC1A mRNA	1.40 ± 0.07 [a]	0.91 ± 0.07 [b]	0.39 ± 0.07 [c]	0.39 ± 0.07 [c]	0.97 ± 0.05	0.57 ± 0.05	< 0.001	< 0.001	0.105
SORBS1 mRNA	0.66 ± 0.07 [a]	0.91 ± 0.07 [ab]	1.14 ± 0.07 [b]	1.12 ± 0.07 [b]	0.97 ± 0.05	0.94 ± 0.05	< 0.001	0.667	0.503
Heat shock proteins									
HSP25/27 mRNA	0.81 ± 0.08 [b]	0.80 ± 0.08 [b]	0.79 ± 0.08 [b]	1.50 ± 0.08 [a]	0.94 ± 0.05	1.01 ± 0.05	< 0.001	0.376	0.746
HSP70 mRNA	0.57 ± 0.08 [b]	0.46 ± 0.08 [b]	0.75 ± 0.08 [b]	1.26 ± 0.08 [a]	0.83 ± 0.06	0.68 ± 0.06	< 0.001	0.077	0.260
HSP90 mRNA	0.87 ± 0.06 [b]	0.76 ± 0.06 [b]	0.86 ± 0.06 [b]	1.29 ± 0.06 [a]	0.90 ± 0.04	0.99 ± 0.04	< 0.001	0.139	0.388
HSP70 protein	0.18 ± 0.25 [b]	0.15 ± 0.25 [b]	0.24 ± 0.25 [b]	2.33 ± 0.25 [a]	0.70 ± 0.18	0.76 ± 0.18	< 0.001	0.816	0.999
HSP90 protein	2.23 ± 0.56 [b]	2.59 ± 0.56 [b]	3.96 ± 0.56 [b]	7.09 ± 0.56 [a]	3.97 ± 0.40	3.97 ± 0.40	< 0.001	0.997	0.273
HSF1 protein	5.33 ± 1.55	4.71 ± 1.55	1.63 ± 1.55	2.17 ± 1.55	3.32 ± 1.10	3.60 ± 1.10	0.282	0.855	0.820

The mRNA expression data are expressed as arbitrary units after normalization with the endogenous reference gene RN18S.
The protein expression data are expressed as arbitrary units after normalization with Coomassie staining.
[a, b, c] Labeled least square means within a row with different letters differ ($P < 0.05$).

Cell viability and development

To evaluate the viability after 72 h of proliferative growth, combined staining was performed with fluorescein diacetate (FDI, marker for living cells) and propidium iodide (PI, marker for dead cells). The viability was affected by the temperature ($P < 0.001$) but not by the pool ($P = 0.242$) or the interaction of both ($P = 0.551$). The viability at 41 °C (92.7 ± 0.6%) was lower than that at all other temperatures (vs. 35 °C: 98.3 ± 0.6%, vs. 37 °C: 97.0 ± 0.6%, vs. 39 °C: 98.5 ± 0.6%, $P < 0.001$ each). In addition, FDI-stained myoblasts (**Fig. 3.2**) were used to determine their size. The cell size was highly affected by the temperature ($P < 0.001$) and pool ($P < 0.001$) but not by the interaction of both ($P = 0.151$). At 35 °C (1525 ± 48 μm^2), the cell size was smaller than that at all other temperatures (vs. 37 °C: 1758 ± 48 μm^2, vs. 39 °C: 1833 ± 48 μm^2, vs. 41 °C: 1943 ± 48 μm^2, $P \leq 0.020$). The cells of pool 5 were smaller than those of pool 20 (1597 ± 34 μm^2 vs. 1932 ± 34 μm^2, $P < 0.001$).

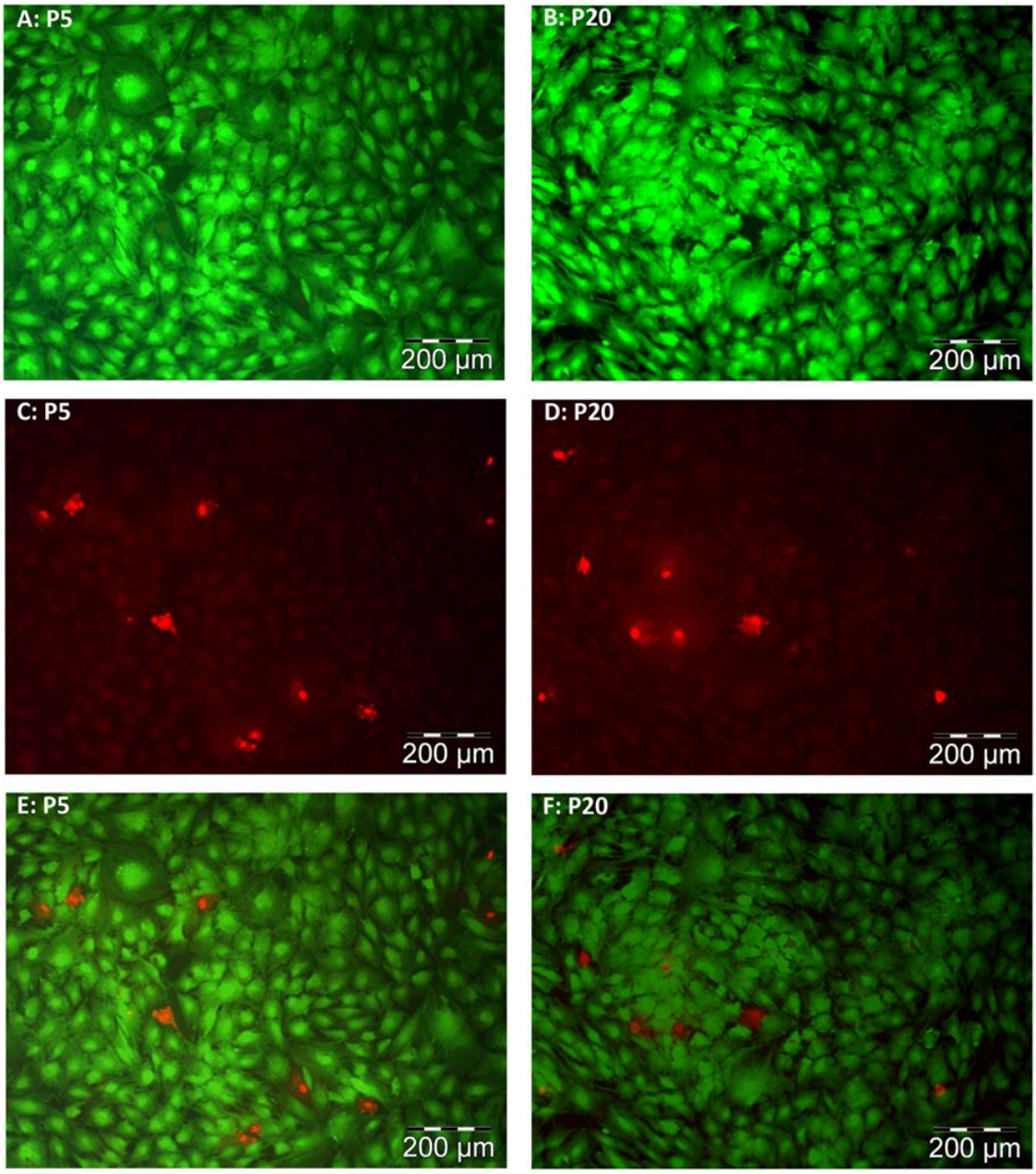

Figure 3.2: Live/dead staining was performed with fluorescein diacetate (FDA) and propidium iodide (PI) for pool 5 (P5) and pool 20 (P20). Viable cells were able to convert nonfluorescent FDA into the green fluorescent metabolite fluorescein because of esterase-dependent conversion (A, B). The nuclei staining dye PI (red) was able to pass through dead cell membranes and intercalate with the cell's DNA double helix (C, D). An overlay of both (E, F) after 72 h of growth at 41 °C is exemplarily shown. For every pool, 30 pictures were analyzed (Nikon Microphot-SA microscope, Nikon Corporation, Tokyo, Japan; Cell^F, Olympus Corporation, Tokyo, Japan)

As shown in **Fig. 3.3** (see also **Additional file 1**, Fig. S6), the myoblasts grown at 35 °C and 41 °C differed in their cellular shapes. Especially after 24 h and 48 h of proliferation, the myoblasts at 41 °C (**Fig. 3.3 D, F**) showed fingershaped protrusions that the cells at 35 °C (**Fig. 3.3 C, E**) did not form.

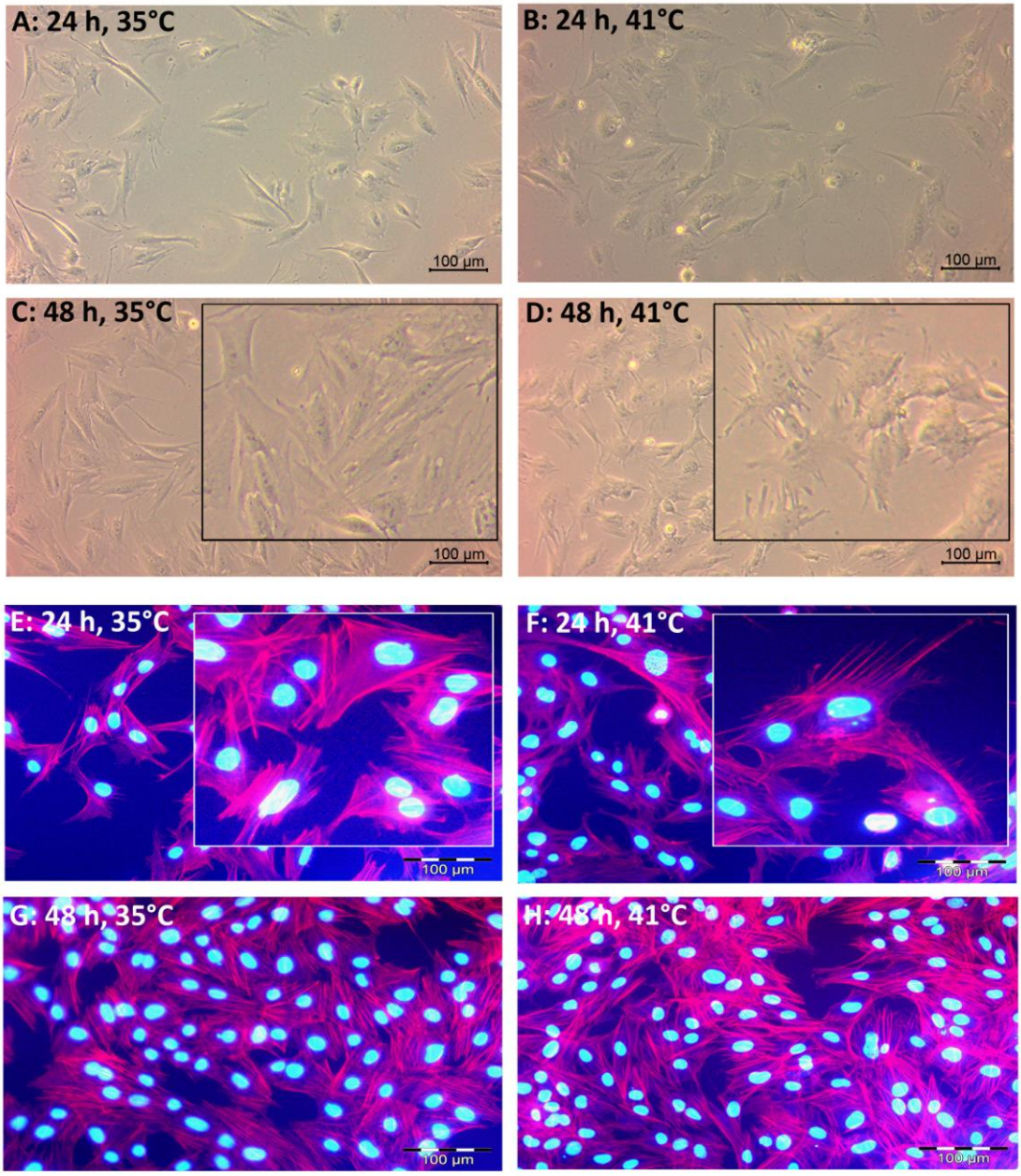

Figure 3.3: Myoblasts derived from satellite cells of M. rhomboideus of 5-day-old piglets were seeded on gelatin-coated dishes and permanently cultivated at 35 °C or at 41 °C for 24 h or 48 h. Images of living cells (A-D) were taken with a Primovert microscope and Axiocam ERc5s (Carl Zeiss AG, Oberkochen, Germany). A staining for actin filaments with Phalloidin CruzFluor™ 594 Conjugate (red) and 4′,6-Diamidin-2-phenylindol (DAPI) for the nuclei (blue) was performed. Images of the phalloidin and DAPI stainings

were taken with Leica DM 2400 fluorescence microscope (Leica Microsystems, Wetzlar, Germany). A higher magnification was presented in the inserts

Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PPARGC1A, **Table 3.3**) is a transcriptional coactivator of energy metabolism. The expression was affected by the temperature and pool (both $P < 0.001$) but not by their interaction ($P = 0.105$). The expression decreased with increasing temperature. In addition, the expression of pool 5 was higher than that of pool 20. Sorbin and SH3 domain containing 1 (SORBS1, also known as ponsin; Table 3) is involved in growth factor-induced signal transduction, cell adhesion, and cytoskeletal organization, and its expression was affected by the temperature ($P < 0.001$) but not by the pool ($P = 0.667$) or the interaction of both ($P = 0.503$). The mRNA expression at 35 °C was lower than that at 39 °C and 41 °C ($P < 0.001$ each), whereas the expression at 35 °C and 37 °C was similar ($P = 0.064$). Myosin-3 (MYH3, also known as MyHCemb) encodes the embryonic isoform of myosin in skeletal muscle. The mRNA expression of MYH3 (**Fig. 3.4 A**) was affected by the interaction between the temperature and pool ($P < 0.001$). In pool 5, the mRNA expression at 35 °C was lower than those at 39 °C and 41 °C ($P \leq 0.002$), and the expression at 37 °C was lower than those at 39 °C and 41 °C ($P \leq 0.004$). In pool 20, mRNA expression was not affected by the temperature ($P \geq 0.915$). In addition, the mRNA expression at both highest temperatures was increased in pool 5 compared to pool 20 ($P \leq 0.005$). Murine small muscle protein X-linked (SMPX, also known as CLS or Chisel) encodes a 9 kDa protein in heart and skeletal muscle cells. The mRNA expression of SMPX (**Fig. 3. 4 B**) displayed the same mRNA expression pattern as that described for MYH3.

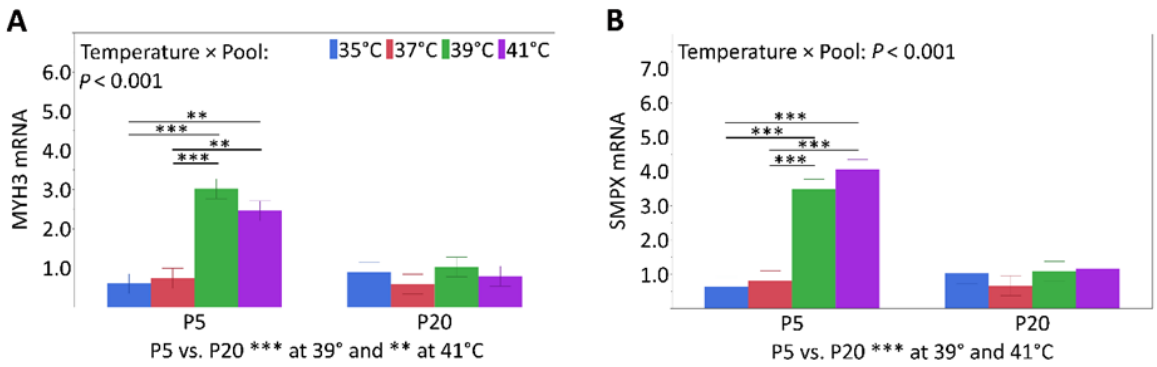

Figure 3.4: Temperature × pool interactions for MYH3 (A) and SMPX (B) mRNA expression analyzed in proliferating myoblasts of pool 5 and pool 20 after 72 h of permanent cultivation at 35°, 37°, 39° or 41 °C. Data (least square means and standard errors) are expressed as arbitrary units after normalization to RN18S expression as an endogenous reference gene. Significant differences within each pool are indicated by asterisks (***P < 0.001, ** P < 0.01), and significant differences between the pools are explained below

Heat shock proteins

The mRNA expression of heat shock proteins (**Table 3.3**) was affected by the temperature ($P < 0.001$) but not by the pool ($P \geq 0.077$) or the interaction of both ($P \geq 0.260$). Higher expression levels of HSP25/27, HSP70 and HSP90 were found at 41 °C than at 35°, 37° and 39 °C ($P \leq 0.002$). The protein expression (see also **Additional file 1**, Fig. S1-S5) of HSP70 (**Table 3.3**) and HSP90 (**Table 3.3**) was affected by the temperature ($P < 0.001$) but not by the pool ($P \geq 0.816$) or the interaction of both ($P \geq 0.273$). HSP70 protein expression was ten times higher at 41 °C than at all other temperatures ($P < 0.001$ each). HSP90 protein expression at 41 °C was increased in the same manner ($P \leq 0.002$). The protein expression of heat shock factor 1 (HSF1, **Table 3.3**) was unaffected by the temperature ($P = 0.282$), pool ($P = 0.855$) or the interaction of both ($P = 0.820$).

mRNA expression of transcription and growth factors

Transcription factors

The mRNA expression of the satellite cell marker paired box 7 (PAX7, **Table 3.4**) and myogenic factor 5 (MYF5, **Table 3.4**) was affected by the temperature ($P \leq 0.022$) and pool ($P < 0.001$), with no interaction between the two ($P \geq 0.426$). PAX7 mRNA expression at 35 °C was higher than that at 41 °C ($P = 0.024$) but not at 37 °C or 39 °C ($P \geq 0.233$). In addition, PAX7 mRNA expression was higher in pool 5 than in pool 20 ($P < 0.001$). Higher MYF5 mRNA expression was found at the lower cultivation temperatures of 35 °C and 37 °C compared to 39 °C ($P \leq 0.047$) and 41 °C ($P \leq 0.009$). In addition, the mRNA expression of pool 5 was higher than that of pool 20 ($P < 0.001$). The mRNA expression of myoblast determination factor (MYOD, **Fig. 3.5 A**) and myogenin (MYOG, **Fig.3.5 B**) was affected by the interaction between the temperature and pool ($P \leq 0.007$). For both genes, there were no temperature-dependent effects in pool 20. For pool 5, higher MYOD mRNA expression was found at the lower cultivation temperatures of 35 °C and 37 °C compared to 39 °C ($P \leq 0.002$) and 41 °C ($P \leq 0.007$). In addition, the MYOD mRNA expression of pool 5 was higher than that of pool 20 ($P \leq 0.015$) at all temperatures. The MYOG mRNA expression of pool 5 at 35 °C was lower than that at all temperatures ($P < 0.001$), and pool 5 exhibited higher MYOG mRNA than pool 20 at 37°, 39° and 41 °C ($P < 0.001$). The mRNA expression of myogenic regulatory factor 4 (MRF4, **Table 3.4**) was unchanged by the temperature ($P = 0.061$) and the interaction of the temperature and pool ($P = 0.291$). The mRNA expression of pool 20 was higher than that of pool 5 ($P < 0.001$).

Table 3.4: mRNA expression of myogenesis-associated genes (least square means ± standard errors) after 72 h of proliferation.

Gene	**Temperature (T)**				**Pool (P)**		**T**	**P**	**T × P**
	35°C	37°C	39°C	41°C	5	20	***P***	***P***	***P***
Myogenic transcription factors									
PAX7	0.92 ± 0.06 [a]	0.76 ± 0.06 [ab]	0.88 ± 0.06 [ab]	0.66 ± 0.06 [b]	0.91 ± 0.04	0.69 ± 0.04	0.022	< 0.001	0.846
MYF5	0.96 ± 0.06 [a]	0.71 ± 0.06 [a]	0.45 ± 0.06 [b]	0.38 ± 0.06 [b]	0.78 ± 0.04	0.47 ± 0.04	< 0.001	< 0.001	0.426
MRF4	1.87 ± 0.19	1.87 ± 0.19	1.67 ± 0.21	0.88 ± 0.29	1.05 ± 0.15	2.09 ± 0.17	0.061	< 0.001	0.291
Growth factors and growth factor receptors									
MSTN	0.85 ± 0.04 [a]	0.69 ± 0.04 [b]	0.42 ± 0.04 [c]	0.37 ± 0.04 [c]	0.70 ± 0.03	0.46 ± 0.03	< 0.001	< 0.001	0.233
IGF2	0.67 ± 0.07 [b]	0.71 ± 0.07 [b]	0.74 ± 0.07 [ab]	1.02 ± 0.07 [a]	1.01 ± 0.05	0.55 ± 0.05	0.015	< 0.001	0.195
EGF	0.79 ± 0.15 [b]	0.71 ± 0.15 [b]	1.09 ± 0.15 [b]	2.42 ± 0.15 [a]	1.27 ± 0.10	1.24 ± 0.10	< 0.001	0.828	0.384
IGF1R	0.89 ± 0.08 [ab]	0.65 ± 0.08 [b]	1.00 ± 0.08 [a]	0.83 ± 0.08 [ab]	0.71 ± 0.06	0.96 ± 0.06	0.043	0.006	0.082
EGFR	1.22 ± 0.10 [ab]	0.88 ± 0.10 [b]	1.36 ± 0.10 [a]	1.40 ± 0.10 [a]	1.06 ± 0.07	1.37 ± 0.07	0.009	0.008	0.165

The mRNA expression data are expressed as arbitrary units after normalization with the endogenous reference gene RN18S.
[a, b, c] Labeled least square means within a row with different letters differ ($P < 0.05$).

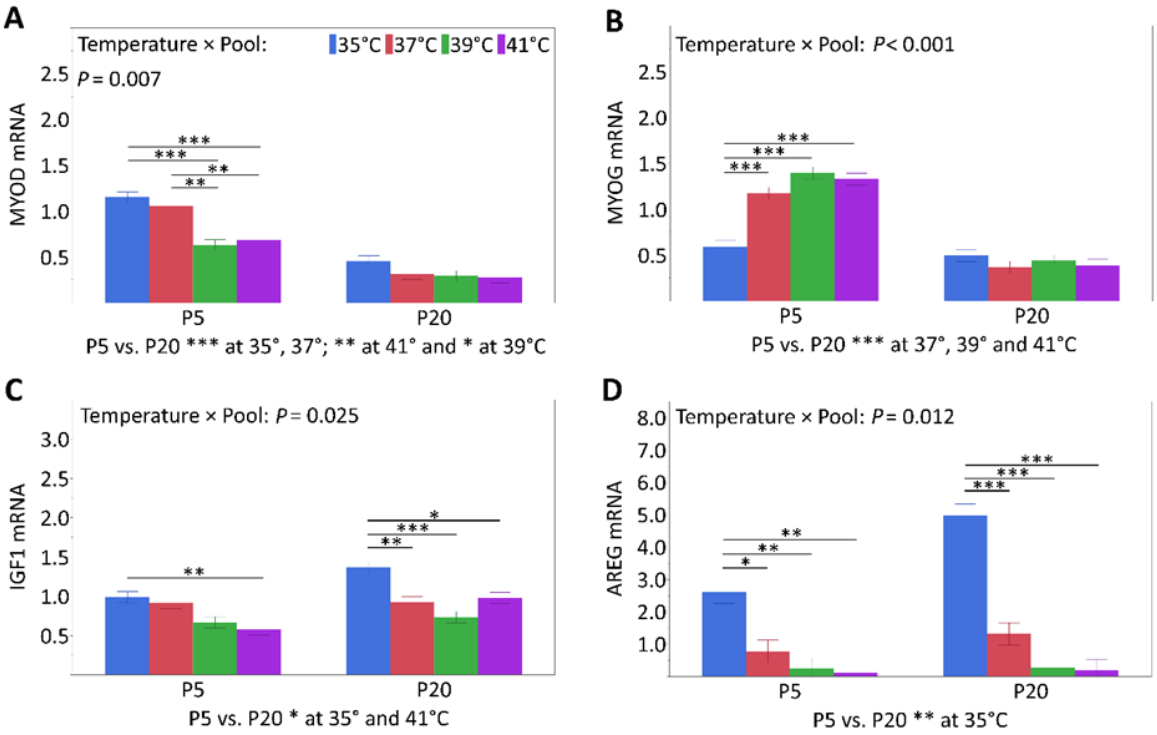

Figure 3.5: Temperature × pool interactions for MYOD (A), MYOG (B), IGF1 (C) and AREG (D) mRNA expression analyzed in proliferating myoblasts of pool 5 and pool 20 after 72 h of permanent cultivation at 35°, 37°, 39° or 41 °C. Data (least square means and standard errors) are expressed as arbitrary units after normalization to RN18S expression as an endogenous reference gene. Significant differences within each pool are indicated by asterisks (*P < 0.05, **P < 0.01 or ***P < 0.001), and significant differences between the pools at the same temperature are explained below

Growth factors and their receptors

The mRNA expression of myostatin (MSTN, **Table 3.4**), a negative regulator of muscle growth, was affected by the temperature ($P < 0.001$) and pool ($P < 0.001$), with no interaction between the two ($P = 0.233$). With increasing temperature, mRNA expression decreased ($P \leq 0.035$), but there were no differences between the highest temperatures (39 °C vs. 41 °C, $P = 0.810$). In addition, the mRNA expression of pool 5 was higher than that of pool 20 ($P < 0.001$). The mRNA expression of insulin-like growth factor 1 (IGF1, **Fig. 5C**) was affected by the interaction of the temperature and pool (P = 0.025). In pool 5, the mRNA expression at 35 °C was higher than that at 41 °C (P = 0.017),

whereas in pool 20, the mRNA expression was higher at 35 °C than at all other temperatures ($P \leq 0.026$). Moreover, the mRNA expression of pool 5 was lower than that of pool 20 but only at the most extreme temperatures (35 °C and 41 °C, $P \leq 0.032$). The mRNA expression of insulin-like growth factor 2 (IGF2, **Table 3.4**) was affected by the temperature ($P = 0.015$) and pool ($P < 0.001$) with no effect on the interaction of both ($P = 0.195$). The mRNA expression levels were higher at 41 °C than at 35 °C and 37 °C ($P \leq 0.037$) as well as in pool 5 compared to pool 20 ($P < 0.001$). The temperature-dependent mRNA expression of epidermal growth factor (EGF, **Table 3.4**, $P < 0.001$) was found with a two-fold increase at 41 °C compared to 35°, 37° and 39 °C ($P < 0.001$). EGF-specific mRNA remained unchanged by the pool ($P = 0.828$) and the interaction of the temperature and pool ($P = 0.384$). The mRNA expression of amphiregulin (AREG, **Fig. 5D**), another ligand of EGF receptor (EGFR), was affected by the interaction of the temperature and pool ($P = 0.012$). For both pools, the mRNA expression at 35 °C was higher than that at all other temperatures ($P < 0.030$). In addition, the mRNA expression at 35 °C for pool 5 was lower than that for pool 20 ($P = 0.003$). The mRNA expression levels of insulin-like growth factor 1 receptor (IGF1R, **Table 3.4**) and EGFR (**Table 3.4**) were affected by the temperature ($P \leq 0.043$) and pool ($P \leq 0.008$) but not by the interaction between the two ($P \geq 0.082$). The lowest IGF1R mRNA expression was found at 37 °C, which was different from 39 °C ($P = 0.030$), whereas EGFR mRNA was lower at 37 °C than that at 39 °C and 41 °C ($P \leq 0.020$). In addition, less IGF1R and EGFR mRNA was expressed in pool 5 than in pool 20 ($P \leq 0.008$).

Discussion

The perinatal period is characterized by drastic impacts of the climatic and nutritional environments in piglets (Schmidt & Herpin 1998). In addition, newborn piglets are naturally exposed to cold but do not possess brown adipose tissue (Trayhurn et al. 1989, Herpin et al. 2002b) and are unable to maintain their body temperature in the first week of life (Curtis & Rogler 1970). Heat stress has a negative impact on livestock production and may influence the growth, animal health and welfare (St-Pierre et al. 2003, Baumgard & Rhoads 2013, Koch et al. 2019). Known results of environmental hyperthermia in pigs

are decreased feed intake, increased heat load, oxidative stress and endotoxemia, among others (Hao et al.2014, Pearce et al. 2015, Ganesan et al. 2018). Previous studies with porcine satellite cells investigated heat shock conditions after precultivation at 37 °C followed by a single high temperature stimulus over a fixed period (Kamanga-Sollo et al. 2011, Gao et al. 2015). For avian satellite cell cultures, a temperature range was considered that included the body temperature and heat stress-inducing temperatures (Clark et al. 2016, Clark et al. 2017, Reed et al. 2017a, Reed et al. 2017b, Harding et al. 2015, Harding et al. 2016). Based on this, our porcine primary muscle cells were permanently cultured between 35 °C and 41 °C with 2 °C incremental temperatures. The standard cultivation temperature for porcine muscle cells is 37 °C, and the temperature challenge with 2 °C below and 2 °C and 4 °C above the standard cultivation temperature is moderate but continually present. To the best of our knowledge, this is the first study of a permanent temperature regime used for porcine satellite cell-derived primary muscle cultures.

for cellular stress (Lewis et al. 1999), remained unchanged. Therefore, both temperatures seem to be physiological temperatures for porcine primary cells (Williams et al. 2008), and some studies preferentially cultivated porcine primary cells at a euthermic temperature of 39 °C (Shim et al. 1997, Xue et al. 2016). The missing effects on the majority of biochemical properties, such as DNA synthesis or LDH and apoptotic properties, also confirmed similar proliferative growth. However, we found effects on the mRNA expression of myogenesisassociated genes between 37 °C and 39 °C that argue for differences in the proliferative growth and differentiation potential at the molecular level. Obviously, the differences were insufficient to induce adaptive changes in the proliferative growth and cellular phenotype. Especially in the case of the early muscle regulatory factors (MRF) MYF5 and MYOD (Rudnicki & Jaenisch 1995), there seems to be a temperature threshold between 37 °C and 39 °C for the reduction of the amount of mRNA as a sign for terminating of proliferation. This fits the higher DNA content and lower PCNA mRNA of muscle cells at 39 °C compared to those at 37 °C. PCNA is a cofactor of DNA polymerase δ whose levels correlate with DNA synthesis, reaching a maximum during the S-phase (Bravo et al. 1987, Baserga 1991). In agreement, culturing primary pig cells at 39 °C enhances cellular processes such as hyperplasia or earlier entry

in the differentiation of stromal-vascular cells (Bohan et al. 2014). The increased mRNA expression of MYH3 and SMPX also indicates differential processes before myotube formation. The latter is known to promote cell signaling-dependent myocyte fusion and cytoskeletal dynamics (Palmer et al. 2001). Moreover, in mice, it was shown that the SMPX gene responded to biomechanical stress (Schindeler et al. 2005).

Effects under permanent cultivation at cold temperature

To the best of our knowledge, this is the first attempt to study the effects of permanent cultivation at temperatures below the physiological range on primary myoblasts. Only studies with turkey or chicken muscle cells used lower temperatures down to 33 °C compared to the control of 38 °C during proliferation but after precultivation at control temperatures (Clark et al. 2016, Clark et al. 2017, Reed et al. 2017a, Reed et al. 2017b, Harding et al. 2016). We found no studies using mammalian primary muscle cells and temperatures below organismic body temperature or usually used cultivation temperatures. Cultivation below the physiological temperature was possible without consequences for the proliferative growth behavior monitored in real time in our study. In addition, due to HSP expression, we conclude that 35 °C did not act as a stressor for myoblasts. However, we found a reduced size of our myoblasts at 35 °C compared to the other (higher) temperatures. Together with the increased DNA synthesis, DNA content and higher amount of PCNA mRNA, this argues for increased proliferative activity, which should normally be detectable by the impedance-based real-time monitoring of the cells. However, we believe that the increased LDH activity as an indication of more damaged and lysed myoblasts could be the reason for the compensation of the effect on CI and the CI-derived parameter slope and doubling time. Due to the comparable myoblast viability at 35 °C and at physiological temperatures of approximately 98%, the increased LDH activity seems to be more a sign of higher proliferative activity than of cell damage at low temperatures. Moreover, we found that the temperature did not affect the apoptotic capability of myoblasts, as formerly shown for chicken myoblasts (Harding et al. 2015). With respect to the mRNA expression of myogenesis-associated genes, it seems that myoblasts cultivated at 35 °C are closer to activated satellite cells and less differentiated (in terms of premyoblasts) than comparable cells cultured at higher temperatures. Higher mRNA expression levels of the satellite cell marker PAX7 and

MYF5 and MYOD also suggest a greater proximity to ancestor satellite cells than to mature myoblasts described after heat stress cultivation (see below). The highest mRNA expression of the PPARGC1A gene at 35 °C argues for the potential thermogenetic activity of muscle cells due to the colder cultivation temperatures. PPARGC1A is known as a transcriptional coactivator involved in mitochondriogenesis and mitochondrial energy metabolism (Quesnel et al. 2019), and the role of muscle tissue as a location for thermogenesis was studied in animals without brown adipose tissue, such as pigs (reviewed in Fuller-Jackson & Henry 2018). For instance, the skeletal muscle of 5- day-old piglets contributes 97% of cold-induced wholebody heat production (Lossec et al. 1998). The reduced mRNA expression of genes involved in differentiation, such as SMPX and MYOG, and in muscle cell structures, such as MYH3, fits with the less differentiated state of myoblasts. The mRNA expression of the EGFR ligand AREG was higher at 35 °C than at the other temperatures. Increased AREG expression was also detected after hypothermia in healthy rat prostate tissue (Kaija et al. 2015). Although the role of specific EGFR ligands in skeletal muscle growth is not clear, EGF could stimulate skeletal muscle growth and differentiation in vitro (Roe et al. 1995, Mau et al. 2008a).

Effects under permanent heat stress cultivation

Our HSP expression results at the mRNA and protein levels clearly showed that 41 °C was sufficient to induce a heat shock response. This is consistent with the results for cultivation at 40.5 °C (Kamanga-Sollo et al. 2011) and 41 °C (Gao et al. 2015) in porcine muscle cells after precultivation at 37 °C. The unaffected HSF1 expression highlights a two-component feedback loop in which HSF1 positively regulates HSP70, whereas HSP70 negatively regulates HSF1 (Krakowiak et al. 2018). In our study, the proliferative ability of satellite cells seemed to end after 72 h at 41 °C. This result is in agreement with other studies using muscle cells derived from Langtang swine (Gao et al.2015) or derived from chicks that were reared at 5 °C higher than standard conditions (Piestun et al. 2017). A rather opposite phenotype of the myoblasts at 41 °C was observed compared to that at 35 °C. Cultivation at 41 °C led to less viability, although the viability after 72 h of cultivation was still approximately 93%. The involvement of apoptosis in heat stress responses of myoblasts is unclear; both our studies and poultry studies (Harding et al. 2015, Piestun et al. 2017) did not find effects on the apoptotic capability, whereas Gao et

al. (2015) found increased apoptosis. The cell size at 41 °C was increased, which was also seen by Gao et al. (2015). Larger cells argue for a higher degree of myoblast differentiation, as described for myocytes (Ganassi et al. 2018). This finding fits with lower MYF5 and MYOD mRNA levels and higher MYH3 mRNA expression at higher temperatures (39 °C and 41 °C). As discussed in the section about physiological temperatures, there seems to be a threshold between 37 °C and 39 °C leading to changes in the mRNA expression. This result is in agreement with lower MSTN mRNA expression at higher temperatures in our study because MSTN (or growth and differentiation factor 8, GDF8) is a negative stimulator of skeletal muscle growth (McPherron et al. 1997). Higher expression at 41 °C was also found for the growth factors IGF2 and EGF. Generally, IGF2 and EGF stimulate both the proliferation and differentiation of muscle cells (Roe et al. 1995, Mau et al. 2008a, Harper & Buttery 1995); reviewed in (Florini et al. 1996, Oksbjerg et al. 2004). We formerly showed that in our porcine muscle cells, IGF2 and EGF mRNA expression increased from proliferating myoblasts to differentiating myotubes (Kalbe et al. 2006). Even the higher SORBS1 expression at 41 °C supported a higher degree of differentiation of myoblasts because SORBS1 is known to be expressed shortly after the onset of myogenic differentiation (Gehmlich et al. 2007, Gehmlich et al. 2010) and marks the establishment of costameres, the cell–matrix contacts. Another protein that is associated with the costameric cytoskeleton, SMPX, was increased during proliferation at higher temperatures (39 °C and 41 °C). This result is in agreement with the hypomethylation of SMPX, indicating upregulated gene expression in pig skeletal muscle after constant heat stress at a 30 °C vs. 22 °C housing temperature (Hao et al. 2016). Moreover, in C2C12 cells, it was shown that SMPX expression is related to the formation of pseudopodia (Schindeler et al. 2005). Pseudopodia are arm-like protrusions filled with the cytoplasm of eukaryotic cells and are used for locomotion. Known types of pseudopodia in muscle cells are lamellipodia and filopodia. Myoblast elongation during differentiation is accompanied by the dynamic extension of filopodia composed of actin filaments to contact neighboring muscle cells before fusion (reviewed in (Pavlath 2010). The increased cell size together with the finger-like protrusions (Fig. 3) at 41 °C argue for a higher degree of myoblast differentiation and a prestage of myoblast fusion.

Effect of the age of the donor piglets

The pools differed in the piglet age at satellite cell isolation by only 15 days of age. However, the peri/neonatal period of piglets is very important for their development. At the age of five days, piglets are thermolabile, whereas those at twenty days of age maintain their body temperature independently (Herpin et al. 2002a). In addition, the muscular alteration of piglets during birth and the first three weeks of age is significant. We formerly showed that the total fiber number in M. semitendinosus was not fixed at birth. The postnatal increase in the myofiber number may be related to both the elongation of existing muscle fibers (hypertrophy) and genesis of tertiary myofibers (hyperplasia, (Bérad et al. 2011)). To our knowledge, this is the first study to consider piglets of different ages in satellite cell isolation, thus accounting for the thermoregulation capacity of donor animals. The effects of temperature were formerly investigated in muscle cell cultures derived from satellite cells of different muscle types in chickens (Harding et al. 2016) or from turkey muscle with different growth rates (Clark et al. 2016). In the current study, we found that both pools differed with regard to most properties, such as the doubling time, cell size and mRNA expression of the majority of myogenesis-associated genes. The MRFs (MYOD, MYF5 and MYOG), which are responsible for myogenic determination or fusion initiation, were expressed at higher mRNA levels in pool 5 than in pool 20, whereas the negative regulator of myogenesis MSTN was expressed in the opposite manner. This speaks for a closer proximity of pool 5 cells to the original satellite cells and because PAX7 was expressed in higher amounts in pool 5. This is in line with other studies on porcine satellite cells that have indicated higher PAX7 mRNA expression after birth and a decrease in the following days (Caliaro et al.2005, Lösel et al. 2013b). We have also postulated the closer proximity to satellite cells for myoblasts cultured at 35 °C. Moreover, the smaller size and the higher mRNA expression of PPARGC1A in pool 5 myoblasts are in agreement with the myoblasts at 35 °C and argue for the less differentiated cells with distinct thermogenetic activity. Significant interactions between temperature and pool were present for six of the investigated genes. For the growth factors, IGF1 (Fig. 5C) and AREG (Fig. 5D) temperature-dependent mRNA expression effects were found in both pools 5 and 20. In contrast, the four myogenesis-associated genes MYOD (Fig. 5A), MYOG (Fig. 5B), MYH3 (Fig. 4A) and SMPX (Fig. 4B) were temperature-dependently expressed only in myoblasts from pool 5 but not from pool 20. Apparently, the primary muscle cell cultures isolated from the satellite cells of the

thermostable donors seem to be less responsive to the temperature changes or even thermal stress for porcine myoblasts, as shown in our study. On the other hand, primary muscle cell cultures isolated from piglets that were labile with respect to their body temperature showed a greater susceptibility to exceeding and falling below a physiological cultivation temperature.

Conclusion

In our study, we used permanent cultivation temperatures above (39 °C and 41 °C) and below (35 °C) the standard cultivation temperature (37 °C) for primary muscle cells of pigs. The porcine muscle cells were able to comparably proliferate at all temperatures regarding their real-time monitored growth behavior. Only the highest cultivation temperature of 41 °C acted as an environmental stressor for the myoblasts, whereas temperatures 2 degrees below and above the standard temperature of 37 °C were not able to induce the expression of HSPs. From our study, we conclude that we consider both temperatures, 37 °C and 39 °C, as physiological temperatures for porcine myoblast growth and differentiation. This result is made plausible by the pigs' body temperature of 39 °C. Cultivation below the standard temperature leads to myoblasts, which are closer to activated satellite cells and less differentiated myoblasts with thermogenetic activity in addition to myogenic determination. Cultivation above physiological temperatures leads to thermal stress in porcine myoblasts and to an acceleration of myogenic development. Myoblasts at 41 °C are larger and have a higher degree of differentiation and finger-like protrusions as a prestage of myoblast fusion. Looking at the age of the cell donor piglets, the adaptive behavior of our primary muscle cells to temperature seems to be determined. Myoblasts derived from satellite cells from thermostable donors at 20 days of age seem to be less responsive to temperature changes or thermal stress than corresponding cells derived from 5-day-old piglets, which are known as thermolabile.

Methods

Cell culture

The isolation of the satellite cells, the establishment of the two cell pools and their validation were carried out as described in detail by Metzger et al. (2020). In brief, two pools were established from M. rhomboideus of 10 female German Landrace piglets at days 5 or 20 of age (pool 5 or pool 20), aliquoted (2 × 106 cells per vial) and stored in liquid nitrogen. *M. rhomboideus* is a mixed muscle with a very high proportion (approximately 75%) of oxidative fibers (Lösel et al. 2013b). The muscle is located in the area of the neck and the shoulder blades and is involved in posture by supporting the head. The percentage of myogenic cells was determined by immunostaining for desmin (D1033, Sigma-Aldrich, Taufkirchen, Germany) with 98 ± 1% desmin-positive cells for pool 5 and 95 ± 2% for pool 20. Proliferative growth was studied over 72 h, including a medium change after 48 h at 35°, 37°, 39° or 41 °C with 37 °C as the standard cultivation temperature. For each temperature, three independent experiments were performed using growth medium (DMEM (Biochrom, Berlin, Germany) supplemented with 0.2ML-glutamine (Carl Roth, Karlsruhe, Germany), 100 IU/mL penicillin (Biochrom), 100 µg/mL streptomycin (Biochrom), 2.5 µg/ mL amphotericin (Sigma-Aldrich), 10% FBS (Sigma-Aldrich), and 10% donor horse serum (HS; Sigma-Aldrich)). For real-time monitoring (e-plate 96, ACEA Biosciences Inc., San Diego, USA), the determination of DNA synthesis (96-well MP, Corning, Wiesbaden, Germany), DNA and protein contents (96-well microplate, Sarstedt, Nümbrecht, Germany), and LDH activity (96-well microplate, Sarstedt), 4000 cells per well from each pool were seeded in 10 gelatin-coated wells. For RNA and protein isolation and live/dead staining and phalloidin staining, 1 × 10^6 cells were seeded on a gelatin-coated 100-mm cell culture dish (Sarstedt).

Real-time monitoring of proliferation

The real-time monitoring of proliferation was carried out with the xCELLigence RTCA SP system (ACEA Biosciences Inc.). Proliferative growth was monitored over 72 h by recording the impedance every 30 min. The data are presented as the CI (arbitrary units),

which corresponds to changes in impedance. For the CI, slope and doubling time data were generated with RTCA Software 1.2.1 (ACEA Biosciences Inc.) as described by Metzger et al. (2020).

RNA isolation, reverse transcription and real-time PCR

RNA isolation, reserve transcription and real-time PCR procedures were previously described (Kalbe et al. 2008, Kalbe et al. 2018) and were performed after 72 h of proliferative growth. Primer information is listed in Table S1 (see Additional file 1, (Lin et al. 2001, Kennedy et al. 1994, Maak et al. 2005, Rehfeldt et al. 2012, Da Costa et al. 2002, Patruno et al. 2008, Jacobs et al. 2006). Data are expressed as arbitrary units after normalization with the endogenous reference gene 18S ribosomal RNA (RN18S). RN18S expression was unaffected by the temperature (P = 0.121), pool (P = 0.281) or their interaction (P = 0.656).

Protein isolation and western blot

After 72 h of proliferative growth, the plates were washed with phosphate-buffered saline (PBS, Biochrom). Then, 400 µL of homogenization buffer at a of pH 7.0 (100mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonicacid (Carl Roth), 250mM sucrose (Carl Roth), 4 mM disodium ethylenediaminetetraacetate (Carl Roth), 1mM dithiothreitol (Carl Roth), and protease inhibitor stock solution according to the manufacturer's instructions (complete Mini, Roche, Mannheim, Germany)) was added and incubated on ice for 5 min. Next, the cells were scraped, collected in a preparation tube (Sarstedt), placed in an ultrasonic bath (Omnilab, Bremen, Germany) of cold water for 2 min, and subsequently centrifuged at 14,000 g and 4 °C for 10 min. The protein content was determined at 280 nm by using the microplate reader Synergy™ MX (BioTek, Bad Friedrichshall, Germany) and the microvolume plate Take 3 (BioTek). The supernatants were aliquoted and stored at − 80 °C. Aliquots of 100 µL of proliferated cells were defrosted. Afterwards, a denaturing protein sample pretreatment was performed by adding blue loading buffer (Cell Signaling Technology, Boston, USA), at 94 °C 4 min. The proteins were separated in a Maxi Buffer tank with gel running buffer (0.12 mol 2-amino-2-hydroxymethyl-propane- 1,3-diol (Tris), 0.97 mol glycine and 0.02 mol SDS) with an electrophoresis

constant power supply (Consort, Turnhout, Belgium). Subsequently, proteins were transferred (60 min, 1.0 mA/cm2) to a PVDF membrane (Carl Roth) with a semidry blotting unit (Peqlab/VWR, Darmstadt, Germany). The SDS-PAGE run time was 1.5 h at 125 mA. Thereafter, the blot was dried at room temperature. Nonspecific binding sites were blocked with skim milk powder in TBST (w/v = 5%) for 1 h at room temperature. The PVDF membrane was incubated with specific primary antibodies against heat shock factor 1 (HSF1, 80 kDa, 12,972, Cell Signaling Technology, Denver, USA), heat shock protein 90 (HSP90, 90 kDa, 60,318–1, Proteintech®, St. Leon-Rot, Germany) and heat shock protein 70 (HSP70, 70 kDa, sc-66,048, Santa Cruz Biotechnology, Dallas, USA) in skim milk powder in TBST (w/v = 5%). The incubation with antibodies was performed with a 1:600 dilution and completed at 6 °C overnight. After washing, the PVDF membrane was treated with the secondary antibody rabbit TrueBlot® anti-rabbit IgG HRP (18–8816, Rockland Immunochemicals, Limerick, USA) in case of HSF1 and mouse True- Blot® Ultra anti-mouse Ig HRP (18–8817-30, Rockland Immunochemicals) for HSP90 and HSP70 at dilutions of 1:50000, respectively, for 90 min of incubation at room temperature, followed by washing three times with TBST and water. For visualization, membranes were developed with SuperSignal® West FEMTO chemiluminescent agent (Thermo Scientific, Schwerte, Germany). Membranes were scanned with a chemiluminescence imager (Intas Science Imaging Instruments, Goettingen, Germany), and band intensities were densitometrically evaluated using LabImage 1D L340 Electrophoresis Software (Kapelan Bio-Imaging, Leipzig, Germany). Western blot analyses were performed once per antibody, and samples of triplicate samples from every temperature of both pools were added per gel (see Additional file 1, Fig. S1). Equal loading of the gels and proper transfer of the proteins to the membranes were verified by Coomassie staining. For staining, the membrane was incubated for 15 min with a solution of methanol (50%, Carl Roth), acetic acid (7%, Carl Roth) and Coomassie Brilliant Blue R (0.1%, Sigma-Aldrich), and a scan followed. The membrane was then removed twice for 5 min (destain solution 1: 50% methanol, 7% acetic acid; destain solution 2: 90% methanol, 10% acetic acid). Data are expressed as the normalized protein abundance after normalization with one band after Coomassie staining (see Additional file 1, Fig. S2). The protein expression of this band was unaffected by the temperature (P = 0.952), pool (P = 0.423) or their interaction (P = 0.817).

DNA synthesis

DNA synthesis was determined after 72 h of proliferative growth by using a commercial colorimetric assay Cell Proliferation ELISA, BrdU (Roche, Mannheim, Germany). The DNA synthesis assay was performed according to Palin et al. (2020), measured by using Synergy™ MX (Bio- Tek), and data are given as the absorbance at 450 nm.

DNA- and protein contents

A combined assay of the DNA and protein contents was established by Rehfeldt & Walther (1997) and adapted for piglet myoblasts by Mau et al. (2008a). DNA and protein contents were measured after 72 h of proliferative growth and were given as μg/well in the monolayers.

Lactate dehydrogenase (LDH) activity

The lactate dehydrogenase (LDH) activity was measured after 72 h of proliferative growth in cell culture supernatants according to the method of Legarnd et al. (1992) as modified by Mau et al. (2008b). After 72 h, supernatants were collected in a preparation tube (Sarstedt) and stored at −80 °C until determination. The LDH activity was expressed as IU/mL of supernatant, the enzyme activity, which converts 1 μM NADH/min/L to NAD at 25 °C.

Apoptosis

The percentage of TUNEL positive cells was detected after 72 h of proliferative growth using the commercial In Situ Cell Death Detection Kit, Fluorescin (Roche). The kit was used according to the manufacturer's instructions, and the TUNEL positive cells were detected with a Leica DM 2400 fluorescence microscope (Leica Microsystems, Wetzlar, Germany) using green and blue fluorescence filters.

Live/dead staining and cell size

After 72 h of proliferative growth, live/dead staining was performed. For this purpose, stock solutions of FDA (5mg of FAD (Sigma-Aldrich) in 1mL of acetone (≥ 99.9%, Carl Roth)) and PI (2 mg of PI (Carl Roth) in 1 mL of PBS (Biochrom)) were needed. The freshly prepared staining solution containing 10 mL of PBS (Biochrom), 16 µL of FDA (5mg/ mL) and 100 µL of PI (2mg/mL) was added to cells washed two times. Then, the cells were incubated in the dark at room temperature for 5min, washed again and analyzed with a Nikon Microphot-SA microscope (Nikon Corporation, Tokyo, Japan) using green and blue fluorescence filters. From every 100-mm cell culture dish, 30 images were taken so that in total 1080 pictures were analyzed. To analyze the cell size, images of green FDA-stained myoblasts were analyzed. From every repetition of each pool, 600 myoblasts were analysed; in total, 14,040 myoblasts were mapped with Cell^F (Olympus Corporation, Tokyo, Japan).

Phalloidin staining

After 24 h, 48 h and 72 h of proliferative growth, cells were fixed with a solution of 4% paraformaldehyde (Carl Roth) in PBS (Biochrom) for 60 min and stored at −80 °C until the staining was performed. For the staining the cultured dishes were defrosted, washed twice with PBS (Biochrom), incubated in the dark at room temperature for 60 min with Phalloidin CruzFluor™ 594 Conjugate (actin filaments, Santa Cruz, Heidelberg, Germany) 1:1000 in PBS (Biochrom) containing 1% bovine serum albumin (Sigma-Aldrich), washed again and for counterstaining ROTI®Mount FluorCare DAPI (nuclei, Carl Roth) was added. Images of the phalloidin (red) and DAPI (blue) stainings were taken with Leica DM 2400 fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Statistical analyses

For statistical analysis, data were subjected to analysis of variance using the MIXED procedure in SAS (Version 9.4, SAS Inst Inc., Cary, USA). The donor piglet age (pool 5 or pool 20), temperature (35°, 37°, 39° or 41 °C), the replication of the experiment (1, 2

or 3) and the interaction of temperature and pool were used as fixed factors. The experiment revealed no significance for all parameters. Differences between the least square means were analyzed with Tukey-Kramer tests. The statistical significance was defined for $P < 0.05$. To ensure a consistent presentation of the results, we have presented the six parameters of the study that showed significant interactions between the fixed factors temperature and pool in figures. For all other parameters, the results are given in tables.

Supplementary Information

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Authors' contributions

Conception and design of experiments: CK and SP; Experimental part and interpretation of data: KM, DD and CK; Data analysis and statistics: KM, AT and CK; Project administration: CK; Writing – original draft: KM and CK; Writing - review and editing: SP, DD, AT, KM and CK. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The animals were obtained from the experimental pig unit of the Leibniz Institute for Farm Animal Biology, Dummerstorf, Germany and were not part of an animal experiment. Animal husbandry and slaughter followed the guidelines set by the Animal Care Committee the State of Mecklenburg- Western Pomerania, Germany, based on the German Law of Animal Protection. Piglets were killed at the FBN slaughterhouse using exsanguination after captive-bolt pistol (5 days of age) or electro stunning (20 days of age). The slaughterhouse is approved by the European Union and the German quality management system QS (MV21212). The isolation of the satellite cells from the dissected porcine muscles, the establishment of the two cell pools used in the presented study were previously described by Metzger et al. 2020.

Consent for publication

Not applicable.

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CHAPTER 4

Based on

The effects of temperature and donor piglet age on the transcriptomic profile and energy metabolism of myoblasts

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The effects of temperature and donor piglet age on the transcriptomic profile and energy metabolism of myoblasts

Abstract

Rapid climate change is associated with frequent extreme heat events and the resulting thermal stress has consequences for the health, welfare, and growth of farm animals. The aim of this study was to characterize the transcriptional changes and the effects on energy metabolism in proliferating porcine myoblasts derived from piglets of different ages, representing differences in thermoregulatory abilities, and cultivated below (35°C) and above (39°C, 41°C) the standard cultivation temperature (37°C). Satellite cells originating from *Musculus rhomboideus* of piglets isolated on days 5 (P5, thermolabile) and 20 (P20, thermostable) of age were used. Our expression analyses highlighted differentially expressed genes in porcine myoblasts cultures under heat or cold induced stress. These gene sets showed enrichment for biological processes and pathways related to organelle fission, cell cycle, chromosome organization, and DNA replication. Culture at 35°C resulted in increased metabolic flux as well as a greater abundance of transcripts of the cold shock protein-encoding gene *RBM3* and those of genes related to biological processes and signaling pathways, especially those involving the immune system (cytokine–cytokine receptor interaction, TNF and IL-17 signaling pathways). For cultivation at 39°C, differences in the expression of genes related to DNA replication and cell growth were identified. The highest glutathione index ratio was also found under 39°C. Meanwhile, cultivation at 41°C induced a heat stress response, including the upregulation of *HSP70* expression and the downregulation of many biological processes and signaling pathways related to proliferative ability. Our analysis also identified differentially expressed genes between cells of donors with a not yet (P5) and already fully developed (P20) capacity for thermoregulation at different cultivation temperatures. When comparing P5 and P20, most of the changes in gene expression were detected at 37°C. At this optimal temperature, muscle cells can develop to their full capacity. Therefore, the most diverse molecular signaling pathways, including PI3K-Akt signaling,

Wnt signaling, and EGFR tyrosine kinase inhibitor, were found and are more pronounced in muscle cells from 20-day-old piglets. These results contribute to a better understanding of the mechanisms underlying the adaptation of skeletal muscle cells to temperature stress in terms of their thermoregulatory ability.

Keywords

Satellite cells, myoblasts, temperature, pig, transcriptome, energy metabolism

Introduction

Climate change exerts multidimensional effects on food and agricultural systems, thereby strongly influencing crop and livestock productivity (Schmidhuber and Tubiello, 2007). Thermal stress may occur under warm and cold environments. This type of stress has led to corresponding hazards due to the increasing number of extreme heat events worldwide, which in turn pose an increased risk to the growth, health, and welfare of animals in farming systems (St-Pierre et al., 2003; Baumgard and Rhoads 2013; Horton et al., 2016). Newborn piglets cannot maintain their body temperature in the first week of life (Curtis and Rogler 1970) due to lack of brown adipose tissue (Trayhurn et al., 1989; Herpin et al., 2002). Changes of the climatic and nutritional environment play an important role during this period (Schmidt and Herpin 1998). Several in vivo studies have investigated the effects of heat stress on the physiology (Le Bellego et al., 2002; Patience et al., 2005; Hao et al., 2014), proteomic profile (Cruzen et al., 2015) and epigenomic profile (Hao et al., 2016) in pigs.

The microenvironment of the myofibres (stem cell niche) largely directs satellite cell functions. The natural environment of the muscle fiber type and its origin play an important role in controlling satellite cell properties (Zhu et al., 2013). Additionally, donor age and species differences can affect the myogenic capacity of satellite cells in vitro (Gonzalez et al., 2020), while the environment can modulate satellite cell sensitivity to thermal stress. Heat stress can affect the differentiation, proliferation, muscle fiber

type, protein turnover, and abundance of heat shock proteins in muscle satellite cells of pigs and chickens as well as in C2C12 myoblasts, an immortalized mouse myoblast cell line (Yamaguchi et al., 2010; Kamanga-Sollo et al., 2011). Muscle metabolism and contractile function are also sensitive to changes in temperature (James 2013). Low temperatures can lead to an energy deficit in skeletal muscle cells, resulting in an increase in mitochondrial biogenesis and ATP production (Jäger et al., 2007; Lira et al., 2010). Relatively few studies have utilized primary muscle cell cultures derived from satellite cells from farm animals to investigate the effects of temperature. Kamanga-Sollo et al. (2011) and Gao et al. (2015) investigated the effects of heat stress by a single high temperature stimulus in porcine satellite cell cultures. Meanwhile, Reed and colleagues investigated the effect of thermal stress (33°C or 43°C vs. 38°C) on the transcriptome of turkey muscle satellite cells at the proliferation (Reed et al., 2017a) and differentiating (Reed et al., 2017b) stages.

We hypothesize that satellite cell-derived cell cultures are able to mimic muscular adaptation to temperature stress as well as exhibit distinct gene expression patterns that reflect their developmental commitment and influence their responsiveness to thermal stress. Therefore, we cultured proliferating myoblasts below (35°C) and above (39°C and 41°C, respectively) the standard cultivation temperature (37°C) and evaluated the effects on the transcriptome, oxidative stress and energetic metabolism, using our well-established cell pooling approach (Metzger et al., 2020). We also investigated the molecular changes occurring in cultures of porcine primary muscle cells originating from donor piglets with different capacities for thermoregulation and cultured under different temperatures.

Materials and methods

Cell culture

The isolation of satellite cells from the *M. rhomboideus* of 10 female five- and 20 days old piglets and the establishment and validation of two muscle cell pools (P5, n = 10;

P20, *n* = 10) were performed as previously described (Metzger et al., 2020). For proliferation experiments cells from both pools stored in liquid nitrogen were defrosted and cultured for 72 h at 35°, 37° (control), 39° or 41°C in growth medium with one medium change after 48 h as described by Metzger et al. (2021). A total of 1×10^6 cells from each pool were seeded in 100-mm gelatin-coated culture dishes (Sarsted, Nümbrecht, Germany) for microarray analysis. To explore mitochondrial and glycolytic functional changes, 2,000 cells/well and 20 wells per pool per replicate (Seahorse XFp plate, OLS, Bremen, Germany) were used. To estimate the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG), 3,000 cells/well and 10 wells per pool per replicate were used (96 well-microplates, Sarstedt). Three replicates were generated for each experiment.

RNA isolation, microarray experiment and analyses

Total RNA was isolated from cells after 72 h of proliferative growth using TRIzol reagent (Sigma-Aldrich) and a RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Porcine Snowball Microarrays (Affymetrix, Thermo Fisher Scientific, Schwerte, Germany) containing 47,880 probesets were used in this study. For cDNA synthesis, 500 ng of total RNA was used and subsequent biotin labelling was performed with the Affymetrix WT plus Expression Kit (Affymetrix) and Genechip WT terminal labeling and hybridization Kit (Affymetrix) according to the manufacturer's instructions. A total of 24 label cRNA samples (*n* = 12 per pool) were hybridized on the microarrays. Afterwards washing and scanning was performed according to manufacturer's recommendations. Quality control was performed using Affymetrix GCOC 1.1.1. software. Expression Console software was used for robust multichip average (RMA) normalization and the detection above background (DABG) algorithm was used to detect the genes that were present. Probe sets with low signal and those that were present in less than 80% of the samples within each temperature group were excluded. After filtering, 13,226 probe sets were finally used for further analyses.

Differential expression analysis was performed using mixed model analysis in JMP genomics (version 9, SAS Institute Inc., Cary, NC, United States). Temperature (35°, 37°, 39° or 41°C), pool (P5 or P 20) and the interaction of pool and temperature were

used as fixed factors. Differences between least square means (LSMs) were analyzed using Tukey-Kramer tests Adjustments for multiple comparisons were performed using the Benjamini and Hochberg (1995), and a corrected p-value threshold of 0.05 was set as the false discovery rate (FDR).

Functional annotation of differentially expressed genes

To identify relevant functional categories across temperatures, pools, and pools under specific temperatures, gene ontology (GO) and KEGG pathway enrichment analysis of differentially expressed genes (DEGs) was performed using WebGestalt 2019 [WEB-based Gene SeT AnaLysis Toolkit (Liao et al., 2019)] and DAVID (v. 6.8). For DAVID, right-sided hypergeometric tests were used to calculate the *p*-values, while dot-plots generated using the R package ggplot2 were used to visualize the DAVID enrichment analysis results. $p \leq 0.05$ was considered significant for biological processes and KEGG pathways.

Validation of microarray results

Quantitative real-time PCR (qPCR) was used for the evaluation of the microarray results. RNA isolation, reserve transcription, and qPCR were performed as described by Kalbe et al. (2008, 2018) with following primers: amphiregulin (*AREG*, Kalbe et al., 2018), myosin heavy chain 3 (*MYH3*, Da Costa et al., 2002), TATA-box binding protein (*TBP*, Erkens et al., 2006), actin beta (ACTB, F - 5′ CTGGCACCACACCTTCTAC - GGGTCATCTTCTCACGGTTG 3′), proliferating cell nuclear antigen (*PCNA*, Metzger et al., 2021), hypoxanthinephosphoribosyltransferase 1 (*HPRT1*, Erkens et al., 2006), heat

shock protein 70 (*HSP70*, Kamanga-Sollo et al., 2011), insulin like growth factor binding protein 5 (*IGFBP5*, Rehfeldt et al., 2012) desmin (*DES*, Wilschut et al., 2008), follistatin (*FST*, Rehfeldt et al., 2012) 18S ribosomal RNA (*RN18S*, Lin et al., 2001) and histidine decarboxylase (*HDC*, D'Astous-Pagé et al., 2017). Normalization of qPCR data was performed with the endogenous reference gene RN18S, which was unaffected by the temperature ($p = 0.121$), by pool ($p = 0.281$) or by the interaction between temperature

and pool (p = 0.656). The LSM ± standard errors (SE) of four genes *AREG*, *PCNA*, *MYH3*, and *HSP70* have been published before (Metzger et al., 2021) and was used for correlation analysis in the present study. Statistical analysis of qPCR data and Pearson's correlation coefficient (r) analysis was performed in SAS v. 9.4 (SAS Institute Inc.).

Bioenergetics assay and ratio of reduced/ oxidized glutathione

Mitochondrial and glycolytic functions were analyzed using the Seahorse XFp Extracellular Metabolic Flux Analyzer, as described in Sajjanar et al. (2019). Mitochondrial function was assessed by the determination of the oxygen consumption rate (OCR, pmol/min/µg of protein), which included nonmitochondrial respiration, basal respiration, maximal respiration, proton leak, ATP production, and spare respiratory capacity. Whereas, the glycolytic functions of the cells were given as extracellular acidification rate (ECAR, mpH/min/µg of protein) including non-glycolytic acidification, glycolytic capacity, glycolysis and glycolytic reserve. The ratio of the reduced glutathione (GSH) and oxidized (GSSG) glutathione was determined by using the GSH/GSSG-Glo^{TM} Assay Kit (Promega, Walldorf, Germany) following the manufacturer's instructions for adherent cells. For statistical analysis, data were subjected to analysis of variance using the MIXED procedure in SAS (version 9.4, SAS Institute Inc.). Pool (P5 or P20), temperature (35°, 37°, 39° or 41°C) and interaction of temperature and pool were used as fixed factors. Differences between the LSMs were analyzed using Tukey-Kramer tests. $P < 0.05$ were considered significant.

Results

The effect of cultivation temperature on the transcriptome

The microarray-based expression profiles of myoblasts after 72 h of proliferation at 35, 39, and 41°C were compared with those cultured at the standard cultivation temperature of 37°C (**Supplementary Table S1**) and the distribution of DEGs was visualized in

volcano plots (**Fig. 4.1**). At 35°C (**Fig. 4.1 A**), a total of 1,683 DEGs were found, 946 of which were upregulated and 737 downregulated. At 39 °C (**Fig. 4.1B**), meanwhile, 1,712 DEGs were identified, 1,023 of which were upregulated and 689 downregulated. Most DEGs (3,178) were found when comparing myoblasts grown under 41°C with those cultured at 37°C (**Fig. 4.1 C**); of these, 1,565 were upregulated and 1,613 were downregulated.

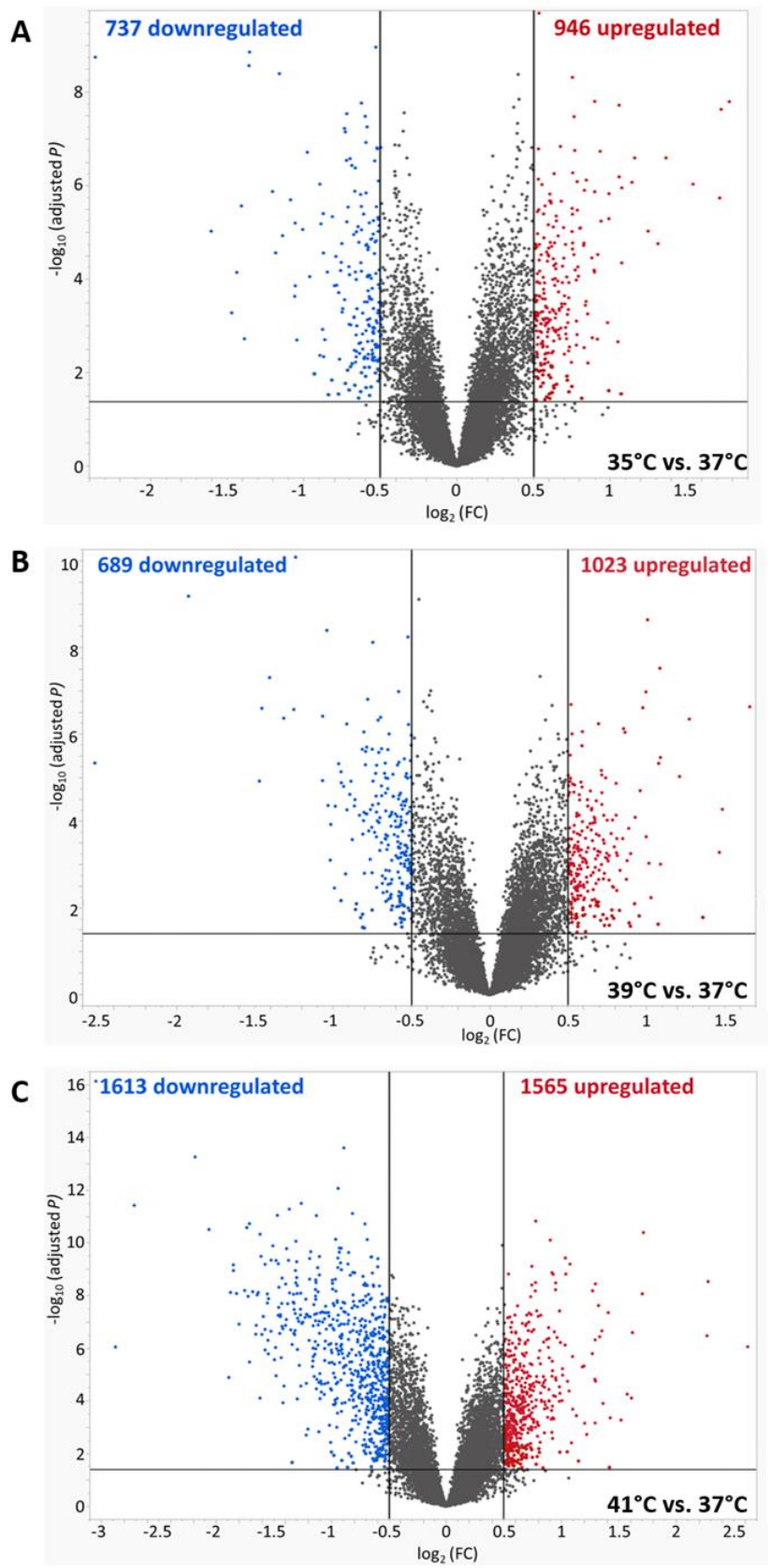

Figure 4.1: Volcano plots of differentially expressed genes (DEGs) of porcine myoblasts after 72 h of permanent cultivation at (A) 35°, (B) 39° and (C) 41°C compared to 37°C. The double filtering criteria are indicated by horizontal (FDR <0.05) and vertical [FC: >

$\log_2$ (0.5) or < $\log_2$ (−0.5)] black lines. Blue dots represent transcripts with lower abundance (downregulated), and red dots with higher abundance (upregulated) at 35°, 39°, and 41°C compared to 37°C.

In addition, 512 overlapping DEGs were found among the different experimental temperature regimes (**Fig. 4.2 A**). For the heatmap (**Fig. 4.2 B**), 11 DEGs were selected that were associated with muscle structure (*DES*, *ACTB*, *LMNA*), proliferation [topoisomerase 2 alpha (*TOP2A*), *PCNA*], immune responses [tumour necrosis factor alpha (TNFA), NFKB1], and prostaglandin biosynthesis (prostaglandin-endoperoxide synthase 2) *PTGS2*, *IGF1*, and *AREG* are growth factors and RNA-binding motif protein 3 (*RBM3*) is a cold-shock marker.

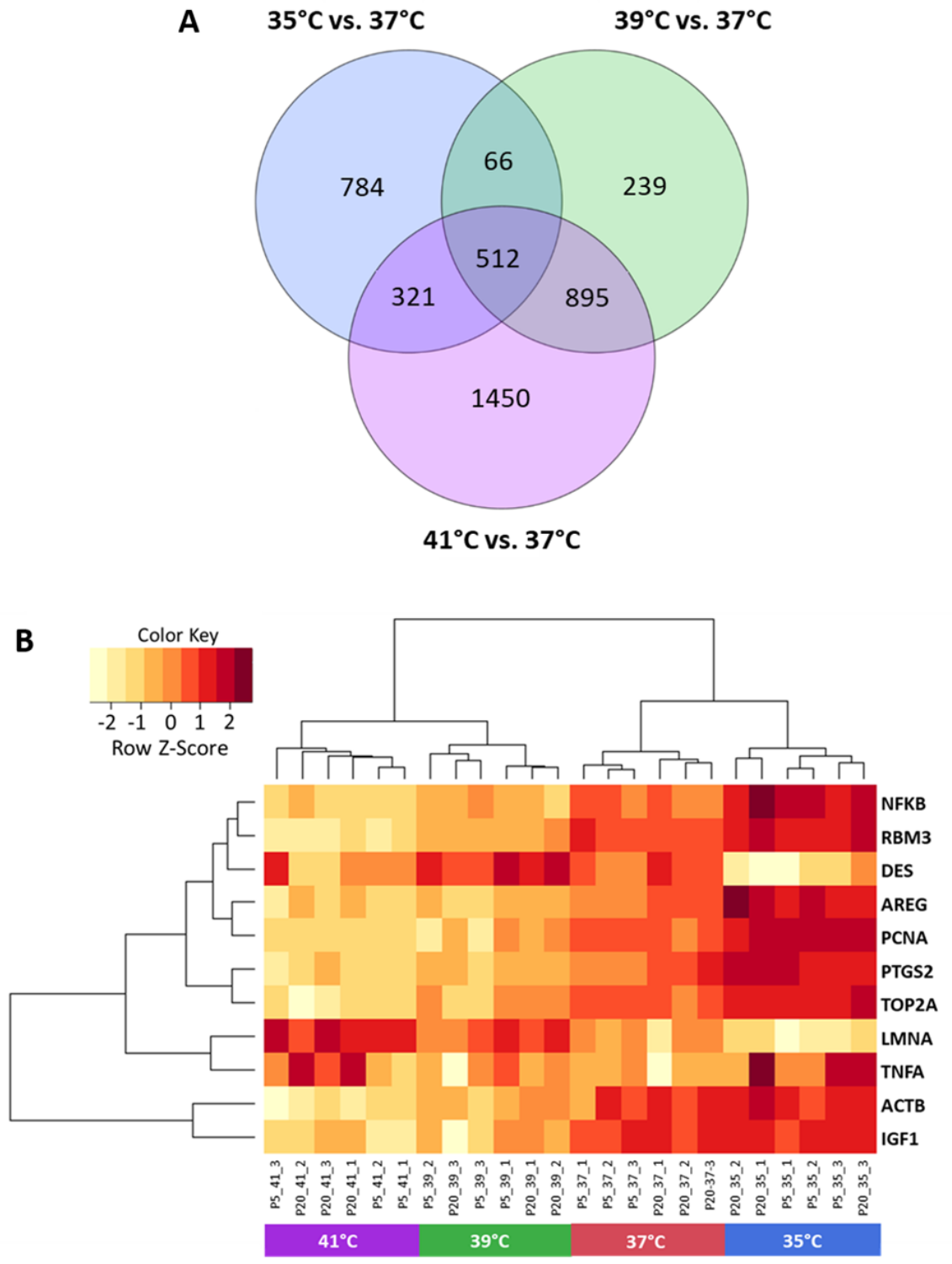

Figure 4.2: Visualization of differentially expressed genes (DEGs) of porcine myoblasts after 72 h of permanent cultivation at 35°, 39°, and 41°C compared to 37°C. Venn diagram (A) shows the number of DEGs for each temperature and the overlapping DEGs between different temperatures (purple 41°C vs. 37°C, green 39°C vs. 37°C and blue 35°C vs. 37°C). Heatmap (B) of 11 DEGs (FDR <0.05) for different permanent cultivation temperatures. The heatmap was generated using hierarchical clustering

method of heatmap.2 function of ggplot2 (version 3.3.5, Wickham 2016) in the R Programming environment (version 4.0.3).

We used DEGs from each comparison of cultured proliferating myoblasts (35, 39, or 41°C vs. 37°C) for GO and KEGG pathway enrichment analysis (**Fig. 4.3 and Supplementary Table S2**). For biological process (BP), the DEGs were found to be enriched in organelle fission, cell cycle, and chromosome organization for all three comparisons. Meanwhile, at the two temperatures above the 37°C reference, the DEGs were mostly associated with the molecular function (MF) of growth factor receptor binding and the DNA packaging complex and chromosome cellular components (CC).

At 35°C, myoblasts showed an enrichment of DEGs associated with the immune response, RNA processing, regulation of immune system process, and regulation of multiorganism process. Specifically, the DEGs in myoblasts cultured at 35°C (low-temperature stress) were enriched in the KEGG pathways of cytokine–cytokine receptor interaction, interleukin 17 (IL-17) signaling pathway, cell cycle, DNA replication, signaling pathway, and TNF signaling pathway. GO enrichment analysis showed that, at 39°C, DEGs were enriched in the biological process of protein-DNA complex subunit organization and the cellular components ribonucleoprotein complex, vesicle, and Golgi apparatus. At 39°C, one KEGG pathway was identified, namely, DNA replication. The most enriched GO terms for the DEGs in myoblasts cultured at 41°C compared with those cultured at 37°C were heterogeneous and included regulation of signaling receptor activity, cell division, regulation of organelle organization, chromosome segregation, response to organic cyclic compound, reproduction, response to lipid, cytoskeleton, signaling receptor regulator activity, and kinase binding. For the highest temperature tested (41°C), three KEGG pathways were prominently represented—DNA replication, cell cycle, and pyrimidine metabolism.

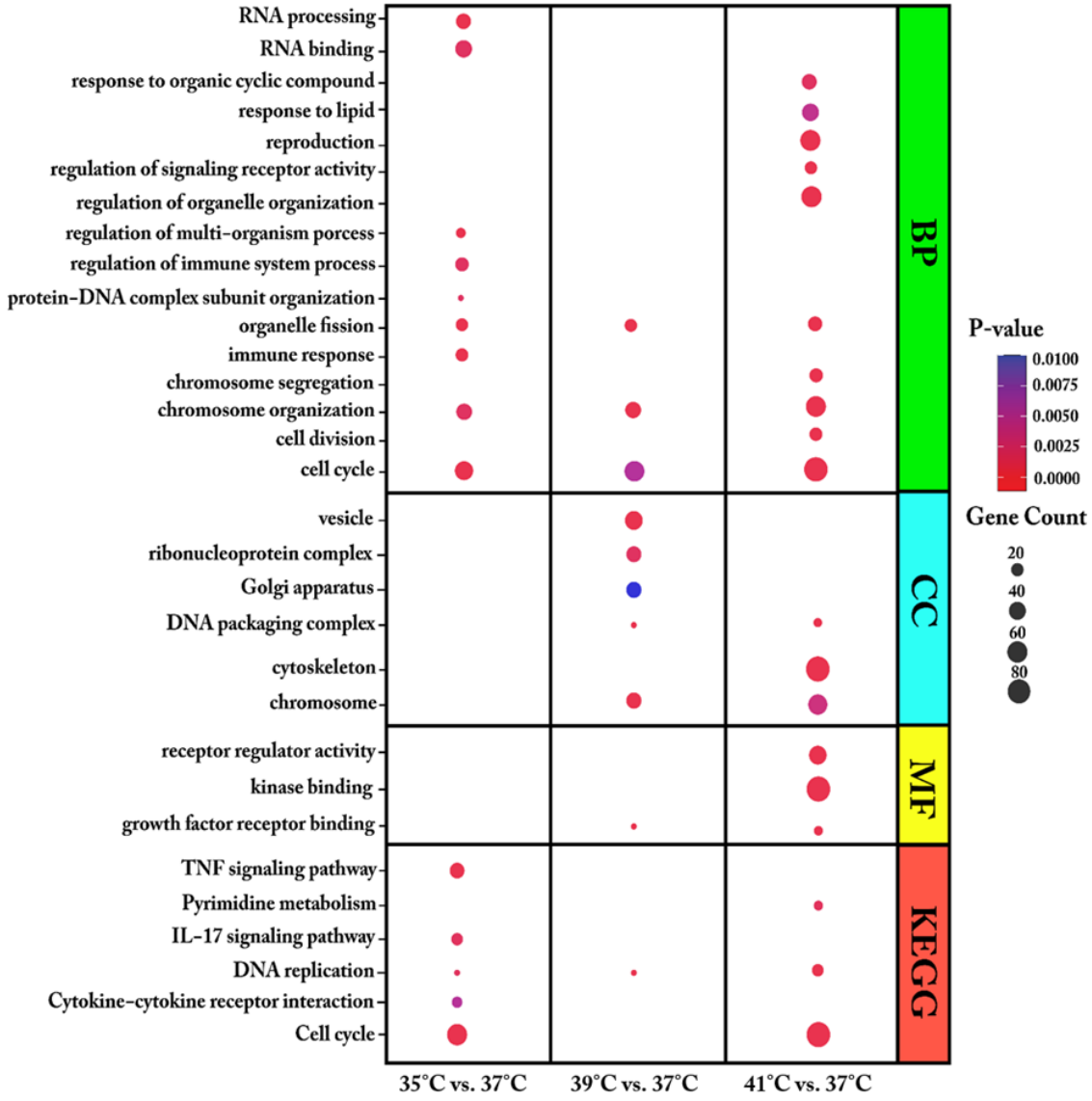

Figure 4.3: Enriched gene ontology (GO) terms of biological process (BP), molecular function (MF) and cellular component (CC) and Enriched Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways assigned to myoblasts after 72 h of proliferation permanently cultured at 35°, 39°, or 41°C compared to 37°C. The dot size embodies the number of transcripts involved in each GO term of biological process (BP), molecular function (MF), cellular component (CC) and KEGG pathway, whereas the dot's color indicates the *p*-value**.**

The effect of donor piglet age on the transcriptome and the interaction of donor piglet age with temperature

A total of 503 DEGs were detected between P5 and P20 after 72 h of proliferation at 35, 37, 39, or 41°C, 340 of which were upregulated and 163 downregulated (**Supplementary Table S3**). For the interaction (Supplementary Table S4) between P5 and P20 at 35°C, a total of 78 DEGs were found, with 40 being upregulated and 38 downregulated. A total of 198 DEGs were detected for the interaction between the pools at 37°C, 145 of which were upregulated and 53 downregulated. For the interaction between the two pools at 39°C, 51 upregulated and 87 downregulated DEGs (a total of 138) were identified. At 41°C, 119 DEGs were found, with 73 being upregulated and 46 downregulated.

When comparing the transcriptomes of the donor cell pools (P5 and P20), the identified DEGs were found to be enriched in key biological processes that included regulation of signal transduction, regulation of protein metabolism, regulation of gene expression, regulation of biological processes, positive regulation of metabolic processes, nervous system development, developmental processes, cell differentiation, and biological regulation (**Fig. 4.4 A, Supplementary Table S5**). Our analysis further revealed several key DEGs enriched in several KEGG pathways, including the Ras signaling pathway, the Rap1 signaling pathway, the PPAR signaling pathway, the PI3K-Akt signaling pathway, glycosaminoglycan biosynthesis, focal adhesion, EGFR tyrosine kinase inhibitor resistance, and ECM-receptor interaction (**Fig. 4.4 B, Supplementary Table S5**). We also undertook a functional annotation analysis of the interaction of culture temperature with the two donor cell pools. Detailed information regarding the biological processes associated with the DEGs for each interaction between pool and temperature is shown in Figure 4A and Supplementary Table S5. Most DEGs between P5 and P20 were found with cultivation under the control temperature (37°C) and were enriched in biological processes such as cellular development, cell differentiation, system development, biological regulation, anatomical structuremorphogenesis, organelle organization, positive regulation of biological process, and developmental process. Interestingly, under cultivation at 39°C, the DEGs between P5 and P20 were also associated with positive regulation of biological process, positive regulation of cellular process, and developmental process. For the interaction of pools at cultivation temperatures above 37°C, the identified DEGs were enriched in the biological processes of signaling and

cellular response to stimulus. For the interaction of pools at the low cultivation temperature (35°C), the genes found to be differentially expressed were enriched in biological processes related to RNA metabolic process, tissue development, and regulation of biological quality, homeostatic process, response to external stimuli, nitrogen compound metabolic process, organelle organization, and gene expression.

Important KEGG pathways affected by the donor piglet age (P5 and P20) under different cultivation temperatures were identified (**Fig. 4.4 B**). For the interaction between P5 and P20 under the cultivation temperature of 35°C, the DEGs were enriched in the ferroptosis and pentose phosphate pathways. Analysis of the interaction of P5 and P20 with culture at the control temperature (37°C) identified pathways associated with EGFR tyrosine kinase inhibitor resistance, viral myocarditis, PI3K-Akt signaling, focal adhesion, PPAR signaling, signaling pathways regulating the pluripotency of stem cells, mTOR signaling, leukocyte transendothelial migration, Wnt signaling, and tight junction. Most transcripts in these pathways were upregulated in P20. For the interaction between P5 and P20 under the cultivation temperature of 39°C, the DEGs were found to be associated with the hypertrophic cardiomyopathy and dilated cardiomyopathy pathways. At 41°C, meanwhile, the DEGs were enriched in the MAPK signaling pathway, the apelin signaling pathway, the PPAR signaling pathway, cardiac muscle contraction, hypertrophic cardiomyopathy, and dilated cardiomyopathy.

Eleven genes were selected for the validation of the microarray data by qPCR (**Supplementary Table S6**). The mRNA expression data are shown in Supplementary Table S6. The 11 genes perform a variety of functions in different molecular pathways in skeletal muscle. MYH3, DES, and ACTB are involved in muscle structure; AREG, IGFBP5, and FST are growth factors or their binding proteins; and HSP70 is a heat shock protein. The TBP, HPRT1, and PCNA genes are associated with mitogenesis and proliferation and the HDC gene is associated with amino acid transport. The microarray and qPCR data showed a high correlation based on Pearson's correlation coefficient (r), as follows: *MYH3* ($r = 0.998$, $p < 0.001$), *DES* ($r = 0.961$, $p < 0.05$), *ACTB* ($r = 0.967$, $p < 0.05$), *AREG* ($r = 0.963$, $p < 0.05$), *IGFBP5* ($r = 0.948$, $p < 0.05$), *FST* ($r = 0.972$, $p < 0.05$), *HSP70* ($r = 0.967$, $p < 0.05$), *TBP* ($r = 0.948$, $p < 0.05$), *HPRT1* ($r = 0.991$, $p < 0.01$), *PCNA* ($r = 0.977$, $p < 0.05$), and *HDC* ($r = 0.977$, $p < 0.05$).

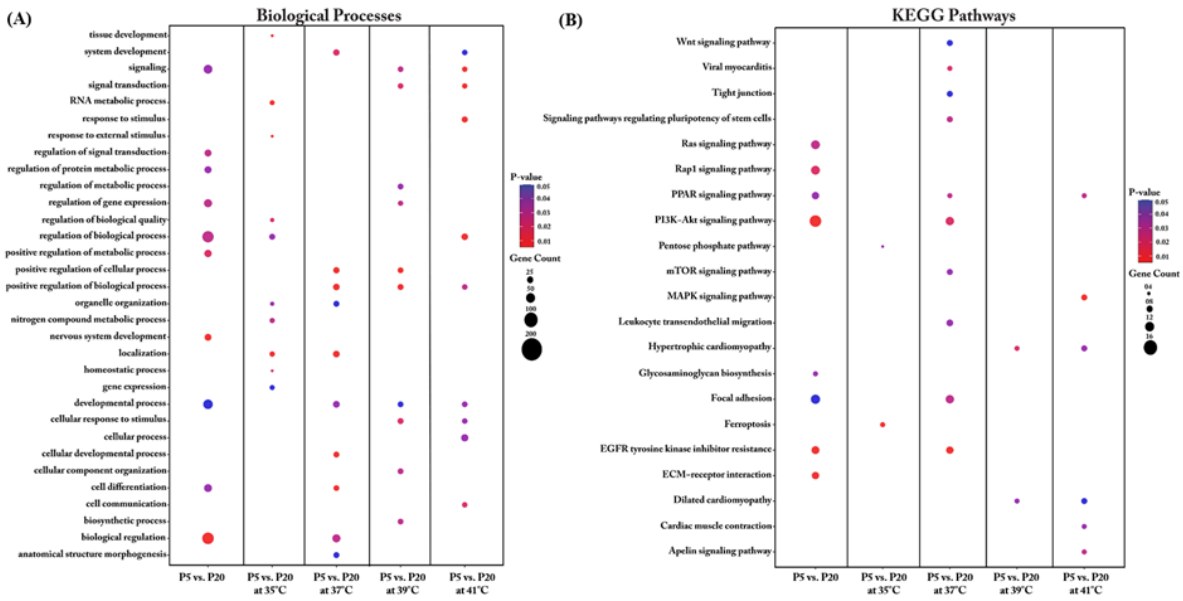

Figure 4.4: Gene Ontology (A) and KEGG pathway (B) enrichment analysis of DEGs between P5 vs. P20 at different temperatures. DEGs between P5 vs. P20 at different temperatures were subjected to DAVID (version.6.8) for functional annotation enrichment analysis. The dot size embodies the number of transcripts involved in each biological process and KEGG pathway, whereas the dot's color indicates the *p*-value.

The effect of cultivation temperature on mitochondrial function

For the evaluation of metabolic flux, we next measured the OCR of the myoblasts (**Fig. 4.5 and Supplementary Table S7**). The levels of non-mitochondrial respiration were affected by temperature ($p < 0.001$) and pool ($p < 0.01$). The highest levels were detected at 35°C ($p < 0.001$ for all comparisons). Additionally, non-mitochondrial respiration levels were higher in P5 (1.053 ± 0.065 pmol/min/µg of protein) than in P20 (0.754 ± 0.064 pmol/min/µg of protein). Basal respiration levels were affected by temperature ($p < 0.05$) but not pool. Basal respiration at 35°C was higher than that at 41°C ($p < 0.05$) but not at 37°C or 39°C. Similarly, maximal respiration levels were affected by temperature ($p < 0.01$) but not pool ($p < 0.10$). The highest respiration levels were recorded at 35°C ($p < 0.05$ vs. all the other groups). Proton leak levels were affected by temperature ($p < 0.01$) but not pool, and were higher at 35°C than at 37 and 41°C ($p < 0.05$ for both), but not at 39°C. ATP production levels displayed the same trend as the proton leak levels ($p < 0.01$). The spare respiratory capacity was unaffected by

temperature or pool. However, spare respiratory capacity at 35°C was higher than that at 41°C ($p < 0.05$). None of the above parameters were affected by temperature/pool interaction.

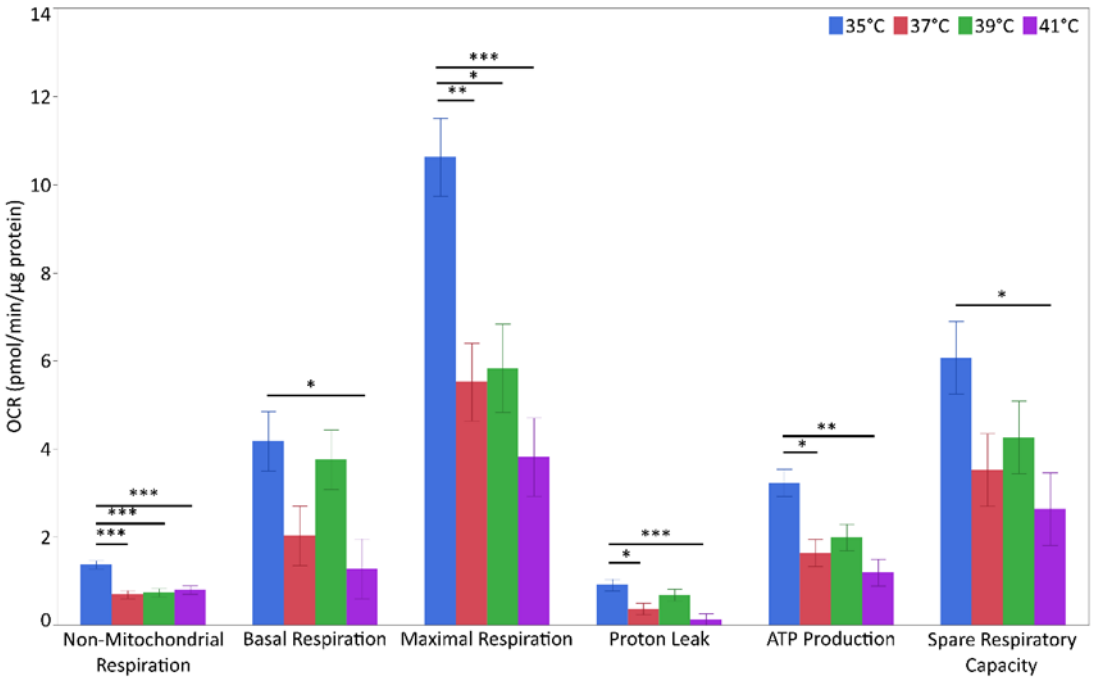

Figure 4.5: Metabolic flux in porcine myoblasts after 72 h proliferation at 35°, 37°, 39°, and 41°C. The non mitochondrial respiration, basal respiration, maximal respiration, proton leak, ATP production and spare respiratory capacity were calculated using the Cell Mito Stress Test Kit. Data (LSM ± SE) were obtained from 10 wells per pool in each of three independent experiments. (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

The effect of cultivation temperature on glycolysis and glutathione levels

For the characterization of glycolytic stress, ECAR levels were measured at different points (**Fig. 4.6 and Supplementary Table S7**). The levels of non-glycolytic acidification were affected by temperature ($p < 0.05$) but not pool or their interaction. The levels of non-glycolytic acidification were significantly higher at 35°C than at 37°C or 41°C ($p < 0.05$) but not 39°C. For glycolytic capacity, glycolysis and glycolytic reserve were unaffected by temperature, pool or their interaction.

GSH is an important scavenger of reactive oxygen species (ROS). The GSH/GSSG ratio is a valuable biomarker of oxidative stress and was found to be affected by temperature ($p < 0.05$) but not pool or temperature/pool interaction. The only difference was found between the two highest culture temperatures, with a higher ratio being detected at 39°C than at 41°C ($p < 0.05$). The results are shown in **Supplementary Table S7**.

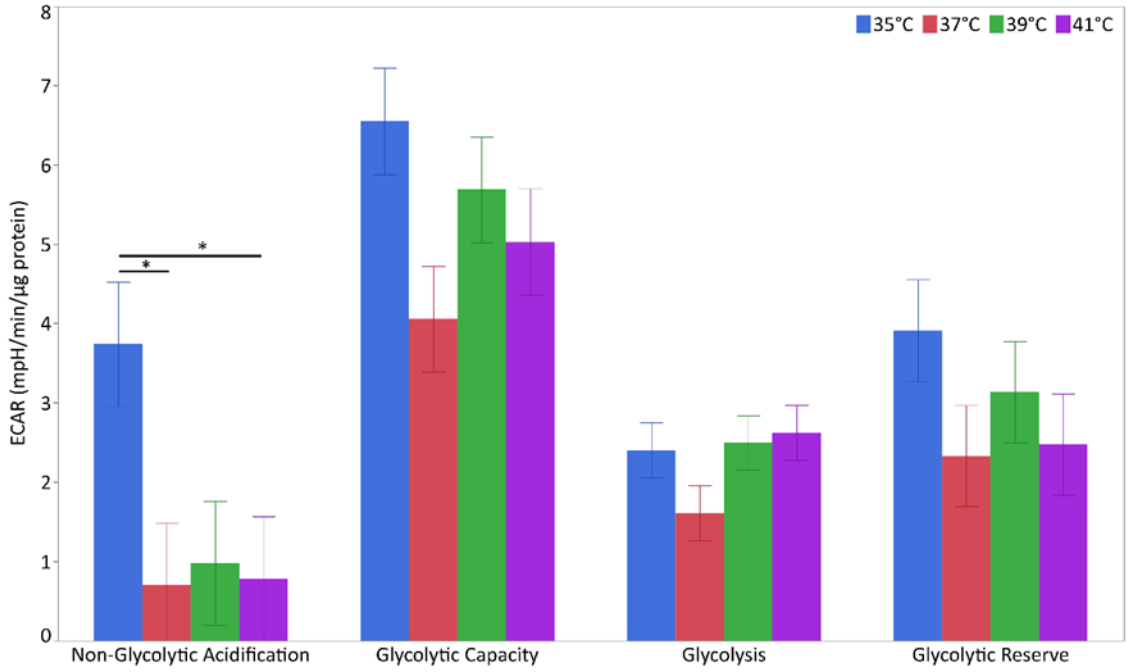

Figure 4.6: Glycolytic flux in porcine myoblasts after 72 h proliferation at 35°, 37°, 39°, and 41°C. The non-glycolytic acidification, glycolytic capacity, glycolysis, and glycolytic reserve were calculated using the Glyco Stress Test Kit. Data (LSM ± SE) were obtained from 10 wells per pool in each of three independent experiments. (*$p < 0.05$).

Discussion

Porcine myoblasts were cultured for 72 h at 35°, 37°, 39°, or 41°C, with 37°C being the standard cultivation temperature, and used as a reference in comparisons. In our previous study, we showed that 37°C–39°C represents the physiological range for porcine primary muscle cell culture. We have previously used cell pooling methods that allow the undertaking of long-term projects involving a wide range of experiments and numerous

replications (Metzger et al., 2020), and this cell pooling method was found to reflect the average proliferative growth behavior of non-pooled cells (Metzger et al., 2020). Accordingly, for this experiment, we used cell pools derived from different animals, although this is a limitation to recognize the biological variability between the different cell donors.

A cultivation temperature of 41°C induces heat stress, whereas cultivation at 35°C results in immature myoblasts (Metzger et al., 2021). In the turkey, a cultivation of proliferating myoblasts and differentiating myotubes below and above the standard cultivation temperature leads to differences in the transcriptomic profiles of the cells (Reed et al., 2017a; Reed et al., 2017b). To the best of our knowledge, this is the first study to examine the effect of temperature stress and the interaction of thermal stress with donor age on the transcriptomic profile and mitochondrial and glycolytic cell functions of myogenic porcine cells.

The effects of cultivation at temperatures below 37°C

Studies involving the culture of primary muscle cells below the standard cultivation temperature are rare. Most have been undertaken using primary muscle cells from birds such as the chicken or turkey (Harding et al., 2015; Clark et al., 2016; Harding et al., 2016; Clark et al., 2017). We have previously shown that porcine myoblasts can proliferate at 35°C but exhibit a different myogenic profile, characterized by higher mRNA expression levels of *PAX7*, *PCNA*, *MYF5*, and *MYOD* and higher rates of DNA synthesis, relative to myoblasts cultured at 37°C (Metzger et al., 2021). This stands in line with the enriched GO terms (cell cycle, RNA processing and RNA binding) in the present study. KEGG enrichment analysis further revealed that DNA replication- and cell cycle-related pathways were affected by cultivation at the low temperature. Additionally, we detected an increase in the levels of *RBM3*, which encodes a member of the family of cold shock proteins (Sonna et al., 2002). Ferry et al. (2011) induced a cold response in C2C12 myoblasts and also observed an increase in RBM3 protein expression. In the present study, we found that desmin (DES) levels were also reduced, and it is known that DES filament formation can be reduced by lower temperature (Chou et al., 1990). Culture of primary pig muscles at 35°C appears to induce an inflammatory response. KEGG

enrichment analysis showed that the DEGs between myoblasts cultured at 35°C and those cultured at the control temperature were enriched in the TNF pathway, IL-17 pathway, and cytokine–cytokine receptor interaction, while regulation of the immune system was enriched as a GO term. TNFA, a pro-inflammatory (Nakano et al. 2006) cytokine, was also highly expressed in myoblasts cultured at 35°C. Other molecular pathways, especially signalling pathways, were also regulated at 35°C. These findings further support those of a previous study, that signaling pathways involved in cell signaling/ signal transduction and cell communication/signal transduction are altered in cold-exposed satellite cells (Reed et al., 2017a).

In addition, we found an upregulation of prostaglandinendoperoxide synthase 2 (*PTGS2*), which codes for a pro/anti-inflammatory enzyme (Funk 2001; Kadotani et al., 2009). PTGS2 is also an oxidation-associated genes and is used as a biomarker for ferrotosis (Yang et al., 2014). The upregulation of acyl-CoA synthetase long chain family member 4 (*ACSL4*) expression is also associated with sensitivity to ferroptosis (Yuan et al., 2016) and also occurs under cold temperatures, as shown in the present study. Guttridge et al. (2000) demonstrated that overexpression of TNFA activates nuclear factor-kappa B (NF-κB) in differentiating C2C12 myotubes. Similarly, in this study, we found that *TNFA* and *NFKB1* expression was upregulated in myoblasts cultured at 35°C. Further evidence that cold exposure stimulates the expression of TNFA in skeletal muscle was provided by Bal et al. (2017). Furthermore, TNFA/NFKB1 signalling in mitochondria was shown to be mediated via autoxidation at complex I or II of the respiratory chain in C2C12 myotubes (Li et al., 1999). Little and Seebacher (2016) showed that murine C2C12 myoblasts cultured at 32°C exhibit higher metabolic flux than those cultured at 37°C. This is comparable to the higher OCR values detected in our myoblasts cultured at 35°C. In addition, we previously (Metzger et al., 2021) showed that the mRNA expression of peroxisome proliferator-activated receptor gamma coactivator 1- alpha (*PPARGC1A*), a known transcriptional co-activator involved in mitochondriogenesis and mitochondrial energy metabolism (Quesnel et al., 2019), was higher in myoblasts continuously cultured at 35°C than in those cultured at 37°C.

The effects of cultivation at temperatures above 37°C

The DEGs between porcine primary muscle cells cultured at 39°C and those cultured under the standard cultivation temperature (37°C) were primarily assigned to the GO terms of cell cycle, chromosome, DNA packaging complex, ribonucleoprotein complex, chromosome organization, protein-DNA complex subunit organization, Golgi apparatus, and vesicle.

The Golgi apparatus contributes to several cellular processes, including mitosis, DNA repair, receptor signaling and cytoskeletal regulation while Golgi-derived vesicles are key components of the intracellular communication machinery (Kalkarni-Gosavi et al., 2019). This was in line with the identified KEGG pathway of DNA replication and was also in agreement with the higher DNA content found in myoblasts cultured for 72 h at 39°C relative to those cultured at 37°C in the present study, as well as the lower *PCNA* mRNA expression levels observed in our previous study (Metzger et al., 2021). Another GO term that was enriched in porcine primary myoblasts cultivated at 39°C compared with those cultured at 37°C was growth factor receptor binding. Higher growth factor receptor expression (epidermal growth factor receptor (*EGFR*) and insulin-like growth factor 1 receptor (*IGF1R*)) with cultivation at 39°C was also found in our former study (Metzger et al., 2021). The products of both genes are known stimulators of DNA replication (Clemmons 1984; Zetterberg et al., 1984; Inoue et al., 2005; Xie et al., 2014), which is in line with the identified KEGG pathway. After culture at 41°C for 72 h, we found that the expression of HSPs was increased, likely as part of a heat shock response, similar to that reported for other studies on porcine muscle cells (Kamanga-Sollo et al., 2011; Gao et al., 2015; Metzger et al., 2021). In addition, beside the higher expression of HSPs, the expression of RBM3 was downregulated (Zeng et al., 2009), which was found in the present study. Furthermore, 41°C seemed to increase the production of reactive oxygen species (ROS) but only compared to the ratio of GSSG/GHS to 39°C. Heat stress can induce mitochondrial superoxide and intracellular ROS overproduction in cultured muscle cells (Rosado Montilla et al., 2014; Kikusato et al., 2015). In addition, after continuous culture at 41°C, biological processes and down-regulated KEGG pathways including pyrimidine metabolism were enriched, which includes all enzymes involved in the synthesis, degradation, salvage, transformation, and transport of DNA, RNA, lipids, and carbohydrates (Garavito et al., 2015). Combined, these findings imply the gradual

termination of myoblast proliferation. Cell cycle arrest after heat stress in porcine primary muscle cells was also found by Gao et al. (2015). In addition, we detected a marked downregulation of *TOP2A* expression in myoblasts cultured at 41°C. TOP2A is a DNA topoisomerase that is associated with RNA polymerase II holoenzyme and is a necessary component of chromatin-dependent coactivation (Mondal and Parvin 2001). Hyperthermia treatment in HeLa S3 cells (15 min at 44°C) resulted in a reduced availability of TOP2A and decreased cytotoxicity (Kampinga (1995), whereas at a later stage in DNA damage processing protection by HSPs overexpression were observed (Li 1987). These observations are in line with our previous study (Metzger et al., 2021) where we found that the expression of HSPs was increased without a concomitant change in the levels of lactate dehydrogenase (LDH), a marker of cell death, after 72 h of proliferation at 41°C. In addition, the lower expression of MYOD at 41°C observed in our previous study (Metzger et al., 2021) was indicative of prominent myoblast maturity. Previous studies also reported that when exposed to heat, myoblasts exhibit changes in the expression of genes related to muscle system development and differentiation (Reed et al., 2017a). Similarly, we identified a GO term of the cytoskeleton with the down-regulation of LMNA, a type V intermediate filament protein. Frock et al. (2005) showed that a reduction in LMNA levels resulted in decreased DES and MYOD expression in primary muscle cell cultures. As mentioned above, we also found that MYOD mRNA expression was reduced in our previous study (Metzger et al., 2021), while *DES* mRNA levels were found to be reduced in the present study. These results support the more differentiated phenotype of myoblasts at the cultivation temperature of 41°C, as evidenced by the presence of finger-like protrusions and an increase in cell size (Metzger et al., 2021).

The effect of donor piglet age and its interaction with temperature

Thermoregulation is the ability to balance heat production and heat loss to maintain body temperature within a certain normal range, which in pigs is between 38 and 40°C, with an average of 38.8°C. Maintaining a neutral thermal environment is among of the most important physiological challenges, especially for newborn piglets. Maintaining body

temperature is most difficult from 0 to 7 days of age because the piglet has no brown fat to quickly generate heat. Accordingly, we used piglets at 5 and 20 days of age, representing donors with a not yet (P5) or already fully developed (P20) capacity for thermoregulation. Understanding the biological effect of temperature stress on muscle cells in aging is important, especially in new-born piglets, which are still sensitive to environmental temperatures. Satellite cell activity was reported to be affected by the origin of donor cells, such as those obtained following maternal nutrient restriction or intrauterine growth restriction (Yates et al., 2014; Raja et al., 2016). Previous study reviewed that skeletal muscle satellite cells derived from different muscle types and different animal selected lines exert differential effects on adipogenesis when thermally challenged (Harding et al., 2015; Clark et al., 2017). Satellite cells isolated from different turkey lines display heterogeneous proliferation and differentiation abilities (Velleman et al., 2000) as well as different sensitivities to temperature changes during proliferation and differentiation (Harding et al., 2015); Reed et al., 2017a). Notably, studies investigating donor age-dependent thermoregulatory capacity remain limited.

Our study also focused on identifying differences in the transcriptomic profiles of porcine muscle cells derived from donor piglets of different ages and continuously cultured at 35, 37 (control), 39, or 41°C. Most of molecular pathways changes when comparing cells of P5 vs. P20 were found at 37°C. At this optimal temperature, the muscle cells can develop to their full capacity and show the most different molecular pathways including PPAR signaling, PI3K-Akt signaling, Wnt signaling pathways and EGFR tyrosine kinase inhibitor. Most of the transcripts enriched in these pathways were more highly expressed in P20 than in P5. However, only small changes between P5 and P20 were detected at temperatures above or below 37°C. We found that the positive regulation of the biological process, the positive regulation of the cellular process, and the developmental process were also found at 39°C, the physiological body temperature of the piglets, when comparing P5 and P20. Interesting, at 35°C, the identified DEGs were enriched in pentose phosphate pathway (PPP) as well as irondependent lipid peroxidation (ferroptosis), which mediates programmed cell death. The glutathione (GSH) system is the main ferroptosis-limiting pathway (Chen et al., 2021). We found significantly lower GSH level in P5 compare with P20 at 35°C ($p < 0.001$). The GSH:GSSG index, an indicator of oxidative stress, tended to be higher in P20 ($p < 0.08$). These results suggested that the muscle cells

of a 5-day-old donor piglet are more susceptible to ferroptosis when exposed to cold temperatures than those of 20-day-old piglets.

Conclusion

In this study, we focused on the transcriptional profile and energy metabolism of primary porcine muscles derived from piglets of different ages (P5 vs. P20) after continuous cultivation for 72 h at 35, 39, or 41°C compared with that at 37°C, the standard cultivation temperature.

Similar patterns of affected GO terms related to organelle fission, cell cycle or chromosome organisation and the KEGG pathway DNA replication were found at the three experimental temperatures compared with cultivation at the control temperature. Cultivation at 35°C stimulated transcriptional responses in immune-related pathways, such as cytokine–cytokine receptor interactions and the IL-17 and TNF signaling pathways. Furthermore, cultivation at 35°C leads to an increase in the expression of RBM3, which encodes a cold-inducible mRNA binding protein, but not a HSP-related response. At 39°C, in addition to cell growth, other GO terms related to protein-DNA complex subunit organization, ribonucleoprotein binding, vesicle, and Golgi apparatus were found to be enriched, suggesting that myoblasts were more developed and more highly structured at this temperature. Only cultivation at 41°C resulted in increased expression of HSPs, indicative of induced heat shock and DNA damage processing responses. The GO terms and pathways associated with pyrimidine metabolism, cell cycle, DNA replication, and cytoskeleton represent the termination of the proliferative ability and cytoskeletal reorganization in porcine myoblast after 72 h of continuous cultivation at 41 °C. When comparing cells from animals of different ages (P5 vs. P20), most molecular changes were found at the control temperature (37°C), which is the optimal physiological temperature. Although only subtle changes in transcript levels were recorded between P5 and P20 at temperatures both above and below 37°C, we nevertheless identified changes in gene expression patterns that reflect the developmental fate of the myoblasts and influence their responsiveness to thermal stress.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2022.979283/full#supplementary-material

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Author contributions

KM: Formal analysis, investigation, visualization, writing–original draft, writing—review and editing. CK: conceptualization, methodology, validation, resources, supervision, writing–review and editing. PS: investigation, writing–review and editing. SP: conceptualization, validation, data curation, resources, supervision, writing–review and editing.

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Data availability statement

The datasets presented in this study can be found in online repositories. The expression data are available in the Gene Expression Omnibus public repository with the GEO accession number (GSE202678: GSM6128337- GSM6128360).

Ethics statement

Ethical review and approval was not required for the animal study because For this study, the animals were used for meat production and underwent no experimental treatment, diagnostic sampling, or any other intervention before killing therefore not requiring specific ethical approval. Animal handling as well as the killing was in accordance with applicable laws, relevant guidelines, and provisions for ethical regulations.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CHAPTER 5

General discussion

General discussion

Between the sarcolemma and the basal lamina of a skeletal muscle, there are myogenic stem cells, or so-called satellite cells (Mauro 1961). For muscle biology studies, the isolation and subsequent cultivation of these cells from myoblasts proliferation to myotube differentiation represents an appropriate *in vitro* model (Molnar et al. 1997). This model is also suitable for large-scale experiments with a large number of replicates in which pooled cells are also used (Mau et al. 2008a, Palin et al. 2020). This is hotly debated, as pooling cells reduces the biological diversity of the cell donors (Stoddart et al. 2012). In addition, this *in vitro* model also offers a chance to investigate the direct effect of various cultivation conditions, such as temperature.

The present work aims to establish two muscle cell pools from the *M. rhomboideus* of thermolabile (5 days old) and thermostable (20 days old) piglets, as a prerequisite for investigating the influence of permanent cultivation temperatures below (35°C) and above (39°C and 41°C) the standard cultivation temperature on proliferative growth. After 72 h of proliferative growth, an endpoint analysis of the physiological parameters, transcription profile, and energy metabolism was performed.

The discussion focuses on the hypotheses identified in Chapter 1.6 of the presented thesis.

Whether the cell pool-based approach is representative compared to individual samples and the advantages and disadvantages of this approach.

The first isolation of porcine satellite cells was performed by Doumit and Merkel (1992). Since then, there have been optimizations of this method, with the aim of increasing yield and purity. The method described in **Chapter 2** is an optimization of the method of Mau et al. (2008b). It is used mostly for the isolation newborn piglets and economically important muscles, such like the *M. logissmus dorsi* of the back or the *M. semimembranosus* of the ham (Mau et al. 2008b, Will et al. 2012, Perruchot et al. 2021). In some studies, porcine muscle cell cultures from less economically important muscle, such as the *M. rhomboideus* (Perruchot et al. 2012, Perruchot et al. 2013), were established. However, the two cell pools used in the present thesis were derived from the *M. rhomboideus* of donor animals with different thermoregulatory capacities, such as

thermolability (5 days of age) or thermostability (20 days of age). This approach has not been investigated before. After isolation, the question was whether it is possible to work with the cells of the individual animals or establish a pool using them. The pooling of cells is still hotly debated because this procedure has disadvantages, such as reduced biodiversity, and statistically, one pool means an n of 1 (Stoddart et al. 2012). In contrast, working with cell pools offers several advantages, such as the possibility for large experimental approaches to explore multiple replicates and a broad spectrum of analyses. In addition, it offers the opportunity to use less donor tissue. Another reason why this issue is debated is the lack of transparency involved in the establishment of the pools. Some studies only mention that cells were pooled, without mention any of the criteria for pooling (Penton et al. 2013). Other studies have established several pools with different numbers of animals or without detailed descriptions of the pooling conditions (Shi et al. 2015, Rubenstein et al. 2020). However, there are studies that described the establishment of muscle cell pools and the determination of their myogenicity (Mau et al. 2008b, Will et al. 2012, Perruchot et al. 2012, Perruchot et al. 2013). **Chapter 2** goes one step further by comparing the proliferative growth and differentiation of three muscle cell pools with those of their corresponding unpooled cells. The muscle cells were derived from piglets/pigs of different ages and genders, as well as from specific skeletal muscles. No differences in proliferative growth and mRNA expression on the part of paired box 7 (PAX7), myoblast determination factor (MYOD), and myogenin (MYOG) were found between the cell pools and their corresponding cells. Moreover, the fusion degree of the differentiating cultures did not differ between the pools and their corresponding donor cells. Taken together, the study supports the approach of using cell pools for the planned experiments with different cultivation temperatures. The following requirements for the use of cell pools from several donors were defined: (1) a detailed data record for the donor's background, including the number of animals and their gender, donor tissue (specific muscle), and birth weight, which is known to strongly affect myogenesis (Rehfeldt & Kuhn 2006) and (2) a detailed description of the isolation and establishment procedures for the cell pools. It is also important to mention that the seeded cell number and developmental stage (cell passage number) for each donor animal should always be equal. (3) At least three experimental replicates are needed to minimize variations.

Whether prolonged exposure to temperatures between 35°C and 41°C with 2°C incremental temperatures are suitable for the cultivation of proliferating myoblast.

The standard cultivation temperature for porcine muscle cell cultures is 37°C (Youg et al. 2007, Kalbe et al. 2008, Baquero-Perez et al. 2012). Rarely have porcine muscle cell cultures been cultivated at different temperatures and, even then, only above 37°C. Only in the work of Kamanga-Sollo et al. (2011) and Gao et al. (2015) were porcine muscle cells cultured at 40.5°C and 41°C, respectively. These studies have aimed to induce a target heat stress. Regarding the occurrence of extreme temperature events, such as heat waves or cold spells (Horton et al. 2016), a permanent cultivation involving mild temperature changes and resulting cellular adaptation should be considered. Primary muscle cell cultures from chickens and turkeys were permanently cultivated in a temperature range of 33° to 43°C (Clark et al. 2016, 2017, Harding et al. 2015, 2016), representing first approaches in farm animals. These studies showed that avian muscle cells are able to grow at all cultivation temperatures. The presented studies in **Chapters 3 and 4** are the first to permanently cultivate porcine muscle cell cultures between 35°C and 41°C, with 2°C incremental temperature increases, for 72 h. The study of **Chapter 3** demonstared by using real-time growth monitoring that different cultivations temperatures do not affect the cell index, its slope, or the doubling time, which suggests that porcine muscle cells can develop their full capacity at all temperatures. Consequently, cultivation temperatures 2°C below and 2°C or 4°C above the standard cultivation temperature are suitable for the proliferative growth of porcine muscle cell cultures.

Whether cultivation temperatures and donor cells representing differences in thermoregulatory capacities have an impact on physiological relevant parameters, signaling pathways or energy metabolism.

The studies presented in **Chapters 3 and 4** are the first to perform a permanent cultivation at 35°C with porcine muscle cell cultures. Only primary muscle cell cultures from chickens or turkeys were cultivated below the standard cultivation temperature (33°C, as compared to 38°C; Clark et al. 2016, 2017; Reed et al. 2017a, b, 2022a, b; Harding et al. 2015, 2016). The study presented in **Chapter 3** showed that 35°C is an adequate

cultivation temperature for proliferative growth and that proliferating myoblasts are smaller and express more PAX7-, proliferating cell nuclear antigen (PCNA)-, myogenic factor 5 (MYF5)-, and MYOD-specific mRNA compared to higher cultivation temperaures. This is evident in the enriched gene ontology (GO) terms on the part of the cell cycle, RNA processing and RNA binding, as well as enriched pathways for DNA replication and the cell cycle, as found in **Chapter 4**. Furthermore, the higher expression of RNA binding protein 3 (RBM3) was found in proliferating myoblasts cultured at 35°C as compared to 37°C. In fact, RBM3 was stimulated by cold, and its overexpression leads to a cold shock response (Hu et al. 2022). Interestingly, no higher expression of known stress markers, such as heat shock proteins (HSPs) were detected. The expression of HSPs is induced by cellular stress, including changes in temperature or osmolality, hypoxia, and free radicals (Diller et al. 2006). Their temperature-dependent response was first described as a signaling response to an elevated temperature. Cells upregulated the expression of chaperone proteins that help fold nascent proteins correctly, refold misfolded proteins, and clear protein aggregation. The overall rates of transcription and mRNA translation were reduced to alleviate the burden of misfolded proteins, while protective genes were selectively expressed, such as heat shock factor 1 (HSF1), heat shock factor 2 (HSF2), and heat shock factor 4 (HSF4) (Åkerfelt et al. 2010). HSFs were translocated to the nucleus, where they activated the expression of chaperones, such as heat shock protein 27 (HSP27) and heat shock protein 70 (HSP70). These HSPs promoted cell survival by inhibiting apoptosis pathways and refolding proteins (Guglizza & Crist 2022). A potential hint regarding the activation of RBM3 and HSPs was provided by Neutelings et al. (2013), in which WI26 cells from a human diploid fibroblast cell line were cultured at 25°C for 5 days and rewarmed at 37°C for 1 to 24 h. The mRNA expression of HSP70 was reduced at 25°C, and rewarming at 37°C resulted in a progressive increase (up to 4-fold after 8 h), followed by a decline. The mRNA expression of RBM3 was increased at 25 °C on Day 1 and returned close to control levels after 24 h at 37°C. These results are aligned with our findings, but for a better understanding of temperature-dependent HSP and RBM3 expression, further studies are needed. In addition, cultivation at 35°C influenced the immune system in the case of an inflammatory response. Therefore, enriched pathways for tumor necrosis factor (TNF), interleukin 17 (IL-17), and cytokine-cytokine receptor interaction and an enriched GO term for the regulation of the immune system were found. This was highlighted by the

overexpression of the pro-inflammatory cytokine TNFA (Nakano et al. 2006), which activated nuclear factor kappa B subunit 1 (NFKB1) (Wullaert et al. 2006), and this mediated autoxidation in the mitochondria (Li et al. 1999). This is aligned with the higher oxygen consumption rate (OCR) values found in **Chapter 4** and the higher expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PPARGC1A), a known transcriptional co-activator involved in mitochondriogenesis and mitochondrial energy metabolism (Quesnel et al. 2019), as shown in **Chapter 3**. Considering all the evidence, permanent cultivation at 35°C leads to smaller cells that are closer to the satellite-cell-activation stage, with an inflammatory response and higher metabolic flux.

There are hardly any porcine cell studies that compare the standard cultivation temperature of 37°C with 39°C. There are only studies that cultivated porcine cells at 39°C. For example, Shim et al. (1997) cultured primordial germ cells, and Xue et al. (2016) cultured pluripotent stem cells derived from *in vitro* fertilization (IVF) embryos at this temperature because this is the body temperature of pigs. In **Chapter 3**, could not any influence on on real-time proliferative growth, apoptosis, or stress response via modified HSP expression be detected. This is consistent with 39 °C being the physiological body temperature of piglets and seems to be a physiological cultivation temperature for porcine proliferating myoblast. Differences as compared to standard cultivation temperature were only found at the mRNA level. Enriched DNA-replication pathway were found in **Chapter 4**, which is aligned with the higher epidermal growth factor receptor (EGFR) and insulin like growth factor 1 receptor (IGF1R) mRNA expression levels described in **Chapter 3** because both are known stimulators of DNA replication (Hellerström et al. 1991, Huang et al. 2013). In addition, higher EGFR and IGF1R mRNA expression levels fit to the enriched GO term for growth factor binding receptor. Furthermore, the researcher found enriched GO terms for cell cycle, chromosome, DNA packaging complex, ribonucleoprotein complex, chromosome organization, and protein-DNA complex subunit organization. This is aligned with the higher DNA content and lower PCNA mRNA expression found in **Chapter 3**. It is known that higher cultivation temperatures can induce mitochondrial superoxide and intracellular reactive oxygen species (ROS) overproduction in cultured muscle cells (Rosado Montilla et al. 2014, Kikusato et al. 2015). The researcher verified an increase of ROS production at the two highest cultivation temperatures, with a higher ratio being detected at 39°C than at 41°C. One potential explanation could be that ROS damage not

only proteins and lipids but also DNA (Schieber & Chandel 2014), and the higher expression of genes related to DNA replication and cell growth was found at 39°C. However, no enriched signaling pathways or other signs of cell death were found. In addition, the lower expression of MYF5 and MYOD mRNA, as well as the higher expression of myosin heavy chain 3 or muscle embryonic myosin heavy chain 3 (MYH3) and small muscle protein X-linked (SMPX), also indicates adaptive processes before myotube formation and seems to influence cell-dependent signaling. The comparison of the standard cultivation temperature (37°C) and piglets' body temperature (39°C) shows similar proliferative growth rates, with differences only at the transcript level for selected genes as a hint regarding the temperature threshold.

Permanent cultivation at 41°C for 72 h leads to a heat stress response. This is aligned with the studies of Kamanga-Sollo et al. (2011) and Gao et al. (2015), which involved inducing heat stress as a punctual temperature peak in porcine muscle cell cultures. As in their studies, a higher expression levels for HSPs at the mRNA and protein levels were found. However, the focus is on the higher expression of HSP70, which is one of the prominent stress markers within this family (Dokladny et al. 2015). Furthermore, cultivation at 41°C does not induce apoptosis, while Gao et al. (2015) found apoptosis in heat-stressed porcine muscle cell cultures at this temperature. Generally, no evidence for programmed cell death at any temperature were found in described studies described of **Chapters 3 and 4**. However, the enriched biological processes and downregulated pathway for pyrimidine metabolism, which include all enzymes involved in the synthesis; degradation; salvage, transformation; and transport of DNA, RNA, lipids, and carbohydrates (Garavito et al. 2015) could indicate this. Specifically, this indicates a gradual termination of myoblast proliferation, which is aligned with the cell cycle arrest found in porcine muscle cells after heat stress (Gao et al. 2015). In **Chapter 3,** ar larger myoblast sizes and lower MYOD expression at 41°C were found, which indicates myoblast maturity. Another research group has confirmed heat-stressed turkey myoblasts exhibit changes in the expression of genes related to muscle development and differentiation (Reed et al. 2017a). In **Chapter 4**, an enriched GO term for cytoskeleton and the downregulation of lamin A/C (LMNA) seem indicate larger myoblast size. In primary muscle cell cultures, the downregulation of LMNA has been found result in decreased DES and MYOD expression levels, as shown by Forck et al. (2005) and in **Chapter 3**. The overall evidence suggests a more differentiated phenotype, one with

increased cell size and characterized by the presence of finger-like protrusions, after permanent cultivation at 41°C compared to 37°C.

The cell pools differed in the thermoregulatory maturities of the donor piglets, whereas they differed in age by only 15 days. The neo/postnatal period is still of great importance, as it is characterized by morbidity and mortality (Carrol et al. 2001, Herpin et al. 2002). The postnatal piglet is the most temperature-sensitive farm animal (Trayhurn et al. 1989), and it takes up to 1 week until its thermoregulation matures (Curits & Rogler 1970). Therefore, one of the cell pools originated from thermolabile piglets (5 days of age, Pool 5), and the other originated from thermostable piglets (20 days of age, Pool 20). The studies in **Chapters 3 and 4** were the first to address different thermoregulatory maturity levels on the part of donor piglets when using primary muscle cell cultures. It has been reported that satellite cell activity was affected by the origin of donor cells, such as those obtained following maternal nutrient restriction or intrauterine growth restriction (Yates et al. 2014, Raja et al. 2016) or those from different turkey lines (Vellemann et al. 2000, Harding et al. 2015, Reed et al. 2017a). The use of primary muscle cell cultures from donors with different thermoregulatory maturity levels to study the effect of different cultivation temperatures is a novel idea.

In **Chapter 3**, an influence on the proliferation of the cells pools were found depending on their thermoregulatory maturation. For example, the cells of Pool 5 were smaller and had a shorter doubling time. In addition, higher mRNA expression levels for MYOD, MYF5, and MYOG were measured, which are responsible for the progress of myogenesis and the onset of differentiation. However, the higher expression levels of myostain (MSTN), as an inhibitor of myogenesis (Lee 2004), and PAX7, as a satellite cell marker, suggest that Pool-5 myoblasts are close to the developmental stage of a satellite cell. This is aligned with the higher mRNA expression for PAX7 in porcine satellite cells after birth, which decreased over time (Caliaro et al. 2005). Myoblasts with a smaller cell size and higher PAX7 and PPARGC1A mRNA expression levels were also observed during cultivation at 35°C, suggesting less differentiated cells were isolated from thermolabile donors or favored by cultivation below physiological temperatures.

Most differences in the transcript profile for Pool 5 and Pool 20 were found at 37°C in **Chapter 4**. This is mainly because, at these optimal temperatures, the cells can develop their full capacity. Different molecular pathways were found, such as EGFR tyrosine

kinase inhibitor resistance, peroxisome proliferator-activated receptors (PPAR) signaling, phosphatidylinositol 3-kinase (PI3K) - protein kinase B (Akt) signaling, and wingless proteines (Wnt) signaling, which were enriched in Pool 20 as compared to Pool 5. In addition, the enrichment of these signaling pathways showed evidence for the increased development of myoblasts in Pool 20 (Yamane et al. 1997, Knight & Kothary 2011, Manickam et al. 2020). Above and below 37°C, only smaller changes between Pool 5 and Pool 20 were detected. At the piglets' body temperature of 39°C, the positive regulation of biological and cellular processes was observed. The differently expressed genes (DEGs) identified at 35°C were enriched in the pentose phosphate pathway (PPP), as well as iron-dependent-lipid peroxidation (ferroptosis), which mediates programmed cell death. The glutathione (GSH) system is the main ferroptosis-limiting pathway (Stockwell & Jiang 2020). This is aligned with the lower GSH level observed in Pool 5 as compared to Pool 20 at 35°C and suggests that myoblasts from thermolabile donors are more susceptible to ferroptosis when exposed to cold temperatures. On the other hand, muscle cell cultures derived from piglets with fully developed thermoregulation were less susceptible to ferroptosis at 35°C. In sum, muscle cell cultures derived from donor animals with fully developed thermoregulation yield more mature myoblasts and are less sensitive to changes in cultivation temperature and thermal stress.

Gaining better insights into working with pooled cells in a model derived from satellite cells offers advantages in the planning of projects in the future. For example, projects in which only a small amount of animal tissue is available can still be implemented, or as in this thesis, a large number of studies and replicates can be carried out. In addition, this thesis showed that this *in vitro* model is very well suited to the selection of donor animals so as to represent the physiological background and imitating the occurrence of extreme temperature events by changing the cultivation temperatures. Thus, new insights regarding the cultivation temperature of porcine muscle cells were provided regarding their capability to achieve proliferative growth at 2°C below and 2°C or 4°C above the standard cultivation temperature. The influence of the donor anmal, specifically its thermoregulatory capacity, was also demonstrated. A pooled muscle cell culture derived from donor animals with mature thermoregulatory capacity was better able to compensate for temperature changes. It was also shown that small changes in cultivation temperature

influence myogenesis *in vitro*. With regard to the more frequent occurrence of extreme temperature events, such as heat waves or cold spells, it is important to understand their influence on the health and welfare of farm animals. Thermal stress also affects animal food yields, which ultimately affect the supply of food to the population. In addition, the quality and safety of these foods is also affected.

CHAPTER 6

Summary

Climate change and the associated occurrence of extreme temperature events have an impact on the health and welfare of farm animals. The temperature-induced stress triggered by both cold and heat has a negative impact on their growth and well-being. This, in turn, affects the supply of food of animal origin, as well as its safety and quality. For example, heat-stressed pigs show a higher proportion of pale, soft and exudative (PSE) meat. This is related to the increased expression of heat-shock proteins in the muscle.

The aim of the present work was to establish an appropriate muscle cell culture derived from satellite cells and investigate the effects of different cultivation temperatures. This *in-vitro* model reflects the ontogenetically interesting developmental stage of the postnatal donor animals. Five-day-old (thermolabile) and 20-day-old (thermostabile) piglets were selected due to their differences in thermoregulatory maturity.

Three studies were conducted to address these issues. The study described **Chapter 2** examined the highly controversial question of whether working with muscle cell pools or the corresponding unpooled cells is preferred. For each of the three cell pools tested, no differences in their proliferative growth behavior were found as compared to their corresponding unpooled cells. The differentiation capacity and mRNA expression of PAX7, MYOD, and MYOG remained unchanged between the cell pools and corresponding unpooled myoblasts. The results demonstrated that the use of cell pools is a suitable approach and reflects the biological diversity of the donor animals. In **Chapter 3 and 4,** the effects of permanent cultivation below (35 °C) and above (39 °C and 41 °C) the standard cultivation temperature of 37 °C were investigated using two cell pools derived from 5-days-old (Pool 5) and 20-day-old (Pool 20) piglets, which had different thermoregulatory maturity levels. In the study described in **Chapter 3**, proliferative growth was monitored in real time, and biochemical and morphological characteristics, as well as hypothesis-based gene expression, were subsequently investigated. The study described in **Chapter 4** examined the temperature-dependent transcript profile and energy metabolism. It was shown that all temperatures are suitable for myoblast proliferation, with no differences in their growth curves being observed. However, differences were found in the cell cultures' biochemical and morphological properties and signaling pathways based on temperature. Myoblasts cultured at 35 °C are smaller

and express more PAX7, while their myogenesis is inhibited, suggesting that they are developmentally closer to an activated satellite cell. In addition, cultivation at 35 °C led to an immune response via the cytokine-cytokine receptor interaction, TNF, and IL-17 signaling pathways, as well as increased metabolic flux. Cultivation at 39 °C, which is also corresponded to the physiological body temperature of the piglets, did not lead to differences in proliferative growth. Compared to 37 °C, higher DNA content and differently enriched genes related to DNA replication and cell growth were detected. Also, differences in the GO terms for protein-DNA complex subunit organization, ribonucleoprotein binding, Golgi apparatus, and vesicle were identified. A heat-stress response was only detected after cultivation at 41 °C, with increased HSP expression at the mRNA and protein levels. Downregulated GO terms for receptor regulator activity, the regulation of signaling receptor activity, cytoskeleton, kinase binding, response to organic cyclic compound, reproduction, response to lipids, and the pyrimidine-metabolism pathway were found. In addition, larger cells with lower mRNA expression levels for MYF5 and MYOD were found, suggesting a more differentiated cell type as compared to those cultured at 37 °C. Most of the differences between Pool 5 and Pool 20 were found at the standard cultivation temperature of 37 °C. This suggests that both pools can reach their full developmental potential at the standard cultivation temperature. In addition, the primary muscle cell cultures reflect the different thermoregulatory maturity levels of the thermolabile and thermostabile donor piglets. The myoblasts of Pool 5 were smaller and expressed more satellite-cell-specific PAX7 mRNA than the myoblasts of Pool 20. The most diverse signaling pathways were found in the myoblasts of Pool 20, including PI3K-Akt signaling, Wnt signaling, and EGFR tyrosine kinase inhibitor resistence.

Future perspectives

The future perspectives derived from this work can be divided into continuing *in vitro* approaches and the global challenge of extreme temperature events, with a focus animal production, health, and welfare. Most of the existing evidence regarding temperature regulation is primarily derived from studies conducted at the cellular-model level. However, it is important to acknowledge the inherent limitations associated with such studies. While cell models provide valuable insights into the cellular mechanisms underlying temperature regulation, they may not fully capture the complexities of the entire organism's response. Mammals possess the remarkable ability to regulate their internal temperature by adjusting and modulating their body temperature. This adaptive process involves both reducing and elevating body temperature in response to varying environmental conditions. In order to comprehensively understand the intricate mechanisms involved in temperature regulation, it is essential to explore the effects of thermal challenges *in vivo.* In summary, while valuable insights can be achieved at the cellular-model level, it is critical for future research to extend investigations to *in vivo* environments and thus gain deeper insights into the regulatory networks and mechanisms responsible for maintaining internal temperature homeostasis in mammals.

The results described in **Chapter 2** show that working with pooled cells is suitable for experiments with a large number of replicates. The cell pools reflect both the proliferative growth and differentiation of their corresponding donor cells. Currently, there are exclusively *in vitro* studies of porcine proliferating myoblasts under thermal stress conditions or in a temperature range from 35 °C to 41 °C. The studies described in Chapters 3 and 4 demonstrate that proliferating myoblasts cultured below or above the standard cultivation temperature have different phenotypes, such as differing cell sizes or transcript profiles, which makes an investigation of differentiating myotube cultures inevitable. Until now, studies of differentiating primary muscle cells under different thermal conditions had only been performed on turkey muscle cell cultures (Reed et al. 2017b, 2022b). In these studies, the muscle cells were more able to differentiate into myotubes at cultivations temperatures of 33 °C or 43 °C as compared to 38°C. Regarding this differentiation, experiments have already been performed our two porcine muscle cell pools, which were cultivated at 35 °C, 37 °C, 39 °C, and 41 °C.

Based on the results shown in **Chapter 4**, cells cultured at 2 °C under the standard cultivation temperature exhibited the higher mRNA expression of RBM3, which is sensitive to cold. The influence of colder ambient temperatures on porcine muscle development in both *in vitro* and *in vivo* studies is not often investigated. As has been shown in the present thesis, even a small temperature drop triggers a temperature response.

The supply of animal protein to the population is a major global challenge, one that is affected by the increased occurrence of extreme temperature events. There are various approaches to meeting this challenge. One is the field of cellular agriculture, in which isolated satellite cells of agricultural origin and tissue technologies are used to cultivate so-called *in vitro* or cultured meat. This is a very contemporary approach, one that also represents a huge challenge (Post 2012, Su et al. 2023). Another approach in farm animal biology is finding better strategies with which to cope with thermal stress. For monogastrics, the search for novel, health-promoting feed additives (e.g., herbs and probiotics) is a promising approach. The aim is to protect the gut barrier from disruption, which can lead to endotoxemia due to heat stress (Abd El-Hack et al. 2020, Ringseis & Eder 2022).

CHAPTER 7

Acknowledgments

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List of abbreviations

acetyl-CoA	Acetyl-coenzyme A
ATP	Adenosine triphosphate
CH_4	Methane
CO_2	Carbon dioxide
CP	Creatine phosphate
DEGs	Differently expressed genes
DNA	Deoxyribonucleic acid
dpc	Days post conception
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ETC	Electron transport chain
$FDAH_2$	Hydroquinone form of flavin adenine dinucleotide
GO	Gene ontology
IGF	Insulin-like growth factor
IGF1R	Insulin-like growth factor receptor 1
LD	*Longissmus dorsi*
MYOD	Myoblast determination factor
MYOG	Myogenin
MRF4	Myogenic regulatory factor 4
MRFs	Myogenic regulatory factors
MYF5	Myogenic factor 5
NADH	Nicotinamide adenine dinucleotide
N_2O	Nitrous oxide
PAX3	Paired box 3
PAX7	Paired box 7
p.p.	postpartum
RH	*Rhomboideus*
RNA	Ribonucleic acid
TCA cycle	Tricarboxylic acid cycle
TNF	Total number of muscle fibers

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