Maintaining the stemness of satellite cells during long-term culture

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Maintaining the stemness of satellite cells during long-term culture

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Maintaining the stemness of satellite cells during long-term culture

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Chapter **1**

General introduction

Meat is one of the most important dietary components in the evolution of man. Animal source foods are suggested to help the evolving man achieve their unusually large and complex brain while simultaneously continuing their evolutionary trajectory as large, active and highly social primates¹. The macronutrients (including different proteins, fatty acids, carbohydrates) and micronutrients (including heme iron, vitamins) in the meat and meat products can provide some key elements in the diet of man²⁻⁴. On the other hand, processed meat and red meat were recently found to have a positive association with several cancers⁵. Notwithstanding, meat is still important for a balanced human food system².

However, the traditional animal agriculture has a substantial contribution to climate change and air pollution, to land, soil and water degradation and to the reduction of biodiversity⁶. Several credible alternatives to livestock meat have been developed. Among them is cultured meat, which should be efficiently produced and should mimic all of meat's physical sensations⁷.

Cultured meat is produced by muscle specific stem cells obtained from a donor animal. Outside of the body, these cells can multiply and form muscle and fat tissue through the process of tissue engineering. It is a technology with the potential ability to solve traditional animal agriculture problems⁸. Even though it still requires time and effort to present cultured meat on the plate of consumers, the technology is available as shown by a proof of concept^{8,9}.

Satellite cells (muscle stem cells) are one of the promising cell sources for cultured meat production^{7,9,10}. Purified satellite cells can proliferate and differentiate into myotubes during in vitro culture¹¹⁻¹⁴ and finally form 3D muscle tissue in vitro^{9,15,16}. However, muscle stem cells will gradually lose proliferation and differentiation ability during in vitro culture^{13,14,17}. Understanding the biological process during cultivation of muscle stem cells and looking for a strategy to maintain the proliferation and differentiation abilities is the major goal of the work presented in this PhD thesis.

1. The challenges of traditional livestock

According to the United Nations, the global population has reached 7.6 billion in 2017 and will reach 9.8 billion in 2050¹⁸. The overall world meat production already reached 317 million tons in 2016¹⁹ and may reach 465 million tons in 2050²⁰, an increase of 45%. This is mostly caused by the rapidly rising incomes and urbanization, combined with underlying population growth in many developing countries²¹. For example, China's total production of raw meat has increased at an annual average rate of 5.8% from 1990-2009. In 2009, China was the world's largest producer of meat with 78.21 million tons²². The predicted increase in demand for meat and other animal products is becoming a big issue for the environment.

Expansion of livestock production causes deforestation and land degradation²⁰. Overgrazing already causes land degradation to some extent in about 20% of the world's pastures and rangelands²⁰. The livestock is also responsible for 18% of greenhouse gas emissions²⁰. Livestock farming accounts for about 9% of man-made carbon dioxide, 37% of methane, 65% of nitrous oxide and about two-thirds of ammonia emissions, respectively²⁰. The livestock sector also accounts for over 8% of global fresh water use²⁰. The unprecedented threat to biodiversity is also partly induced by the expanding livestock sector²⁰. Altogether, the current livestock sector is not environmentally sustainable and would be even less so in the future.

The increasing concern about animal welfare is another problem in livestock. Because of the gap between the meat production and consumption, animal farmers paid more attention to economics and yield per animal, then to animal welfare and animal behavior^{7,23}. However, welfare-friendly products are more popular and consumers are willing to pay more (e.g.: free range vs caged egg)²³. Thus, public concern about animal welfare may change the animal production practices in the future^{7,23}.

Human and livestock health is also a common concern in the livestock sector. On one hand, livestock provide a full complement of nutrients in human diets; on the other hand, processed meat and red meat are found to have a positive association with several cancers, coronary heart disease and type 2 diabetes^{2,4}. The overuse of veterinary drugs and antibiotics also threaten human health through the rise of antibiotic-resistant strains of micro-organisms⁶. In addition, the frequent eruptions of animal diseases can reduce production and productivity, disrupt local and national economies, threaten human health and exacerbate poverty^{21,24}.

The imbalance between a growing livestock meat demand and limited natural resources drives the action towards far-reaching change. One of the actions is to look for efficiency gains in resource use, promoting industrialization of livestock production²⁰ and reducing waste of food²⁵. Another way is to reduce meat calories by using alternative sources of protein^{7,19}, such as plant and insect protein^{7,26-28}. Cultured meat is a potential technology, which can partially solve major upcoming problems by traditional livestock^{7,8,29}.

2. The history of cultured meat

Winston Churchill wrote in his book *Thoughts and Adventures* (1932) "...Fifty years hence, we shall escape the absurdity of growing a whole chicken in order to eat the breast or wing, by growing these parts separately under a suitable medium..."³⁰. With the development of knowledge in muscle development^{31,32}, tissue engineering³³⁻³⁵ and stem cell biology^{11,36-38}, cultured meat has been proven as a concept in 2013⁹. Table 1 lists the events in cultured meat history.

Table 1. Events in cultured meat history

Time	Event
1930s	Winston Churchill suggest growing chicken meat under a suitable medium. ³⁰
1960s	Description of muscle development ³⁹ . Embryonic muscle cells can proliferate and differentiate in vitro.
2000	Bio-artificial muscles (BAMs) produced from the skeletal muscle resident stem cells for medical usage ³³ .
2002	America National Aeronautics and Space Administration (NASA) supported research on cultured gold fish for space missions ⁴⁰ .
2001-2007	The Dutch government supported cultured meat research at Amsterdam University, Utrecht University and the Eindhoven University of Technology ⁴¹ .
2004	New Harvest is established in 2004, aimed at building the field of cellular agriculture including cultured meat.
2008	The first “In vitro Meat Symposium” in 2008, held in Norway.
2013.08	Professor Mark Post presented the first cultured beef hamburger in London, 2013. It is the proof of concept that cultured meat could work ⁹ .
2015.10	Mark Post and Peter founded the cultured meat company MosaMeat B.V. in the Netherlands.
2015	Uma Valeti and Nicholas Genovese founded the cultured meat company Memphis Meats in 2015 in America.
2016 until now	More than 30 companies already founded to start cultured meat research.

3. The basic structure, composition, quality of meat

After slaughtering, the carcass contains muscle tissue, fat tissue, connective tissue and bone. About 50-60% is muscle tissue and this is the most important part for raw meat and meat processing⁴². Skeletal muscle tissue consists of approximately 90% muscle fibers and 10% connective and fat⁴³. The basic structure of muscle tissue has been clearly characterized⁴⁴. The general structure of muscle is separated by three basic membranes. The endomysium separates the individual muscle fibers, the perimysium delineated fascicles within the muscle and the epimysium encases the entire muscle⁴⁵. The blood vessels, major nerves and extracellular matrix support muscle function by providing oxygen, energy, contraction stimuli and mechanical support. The general

composition of muscle is 75% water, 19% protein, 3.5% of soluble, nonprotein, substances including inorganic compounds, and 2.5% of fat⁴⁶. The proteins can be divided into the sarcoplasmic proteins, the myofibrillar proteins, the proteins of the connective tissue and other structures⁴⁶. Fat tissue is another important tissue in the carcass. Depending on species and anatomical location of the muscle, adipose tissue accounts for about 3%-45% of the carcass, and intermuscular fat content generally ranges from 1% to 11%^{4,47}. Connective tissue accounts for about 10% to 20% of the carcass and intramuscular connective tissue accounts for about 10% of skeletal muscle^{46,48}. It is mainly composed of collagen, elastin and reticular protein.

The sensory quality of meat during eating is determined by color, tenderness, flavor, water-holding capacity, juiciness and taste. Meat quality is strongly affected by muscle composition such as protein, fat and connective tissue. The sarcoplasmic protein myoglobin is the heme containing molecule that is responsible for meat color⁴⁹. For instance, tenderness of meat is determined by intramuscular proteins such as structural and non-structural proteins, proteins in connective tissue, but also by the amount of intramuscular fat⁵⁰. The peptides and amino acid content, free fatty acids as well as nucleotides are contributing to the overall flavor and are appreciably modified during cooking and meat processing⁵¹. The degradation of thiamine (vitamin B1), glycogen, sugars, and organic acids during heating also affects meat flavor⁵¹. Water-holding capacity is related to the structural and sarcoplasmic proteins in the muscle, but also to the fat content and strongly determines juiciness⁵². For cultured meat to be a credible mimic of conventional meat, these specific qualities should be considered.

4. The potential production way for cultured meat industry

As the producer of the world's first cultured beef item, Mark Post described the process to make the burger⁹. They took a biopsy from a cow and retrieved the satellite cells. The cells were expanded in a traditional cell culture system requiring culture medium and serum, until a sufficient number of cells ($> 10^{10}$) was obtained. In packages of about 1.5 million, cells were then taken up in a collagen/Matrigel™ gel that was deposited in a culture dish around a central column of agarose gel. In this environment, the cells self-organized into a donut-shaped muscle fiber of 1mm thick and matured towards a protein-rich muscle fiber as a result of contraction, compaction and the development of tension. After 3 weeks this maturation process was complete and the muscle fibers were harvested. For the 85 g of the world first cultured hamburger, they used 10,000 of these muscle strips⁹.

Van der Weele C and her colleagues summarized the potential flow chart of cultured meat industry production⁵³.

Figure 1. A potential production process for cultured meat

5. Cell sources for cultured meat

In the field of biomedicine, the isolation and expansion of muscle stem cells^{11,13,14}, and differentiation in three-dimensional conditions^{54,55} are now feasible at laboratory scale. However, turning cultured meat into a commercial product still requires additional technical efforts to improve and complement the product and make it scalable, efficient and cost-effective⁸.

Cultured meat is based on cell culture. There are several requisites for good cell

candidates. The cells should be easy to harvest and have substantial expansion and differentiation capacity in vitro. The serum-free/chemically defined medium needs to be available for this cell type or one should be able to develop this. The differentiation ability to myotubes should be relatively high for those cells. They should be genetically stable after multiple divisions.

5.1 Other cell sources and their advantages and disadvantages

Several cell types have the potential to form myotubes and muscle fibers and are therefore candidates for cultured meat production. Embryonic stem Cells (ES) or induced pluripotent stem cells (iPS) have the ability to differentiate into any cell type and can propagate seemingly interminable⁵⁶⁻⁵⁹. In addition, there are reported methods to culture these cells under xeno-free and chemically defined conditions⁶⁰. However, the method to induce ES/iPS to myoblasts or myotubes is still complicated and not very efficient^{59,60,61} and protocols for livestock animals are still rare⁶².

Mesenchymal stem cells (MSC) can be extensively expanded and they are multipotent, i.e. capable of differentiating into various lineages, notably fat, bone and cartilage^{63,64}. MSCs can also expand in commercially available xeno-free and chemically defined medium^{63,65}. However, the myogenic differentiation of MSC is relatively low⁶⁴. It remains to be determined if this is species and origin dependent.

A host of other cells have reportedly potential to form myotube, such as myoendothelial cells, stem cell biomarker CD133+ cells, cell stress mediator PW1 positive interstitial cells (PICs), muscle-derived stem cells, Twist2⁺ progenitor cells, and muscle side population cells. However, All of myoendothelial cells, CD133+ cells, PICs, muscle-derived stem cells and muscle side population cells contain the satellite cell population⁶⁶⁻⁷¹. Even though the TWIST2⁺ cells are distinct from PAX7⁺ satellite cells in vivo, they quickly upregulate PAX7 and MYOD expression after culturing in vitro, which suggesting TWIST2⁺ cells are the progenitor cell of myoblast⁷². These results suggest that the myogenesis ability of these cells are from the satellite cells or myoblast cells when cultured in vitro^{69,71}.

5.2 Satellite cells as the cultured meat cell source

The muscle stem cells (MuSCs; also known as satellite cells) are a heterogeneous population of adult stem cells located in skeletal muscles^{11,73-75}. They were first identified by Mauro in 1961³⁸. These cells reside between the muscle sarcolemma and the basal lamina of muscle fibers and remain quiescent under normal conditions^{11,73,76,77}. Upon injury, quiescent satellite cells are activated to repair muscle injury while a subset of the activated satellite cells return to the quiescent status after self-renewal^{11,73,78}. Satellite cells are essential for muscle regeneration and mice lacking satellite cells display severe muscle regeneration defects^{79,80,81}. Satellite cell impairment occurs in muscular diseases such as Duchenne muscular dystrophy (DMD)⁸² and in aged muscles⁸³.

Figure 2. Regulator markers for quiescent satellite cells, activated satellite cells, myoblasts and myocytes. Adapted from Norio Motohashi and Atsushi Asakura⁹⁴.

Several surface markers are identified for satellite cells in mice and human. Mice satellite cells express vascular cell adhesion molecule 1 (VCAM1), C-X-C chemokine receptor type 4 (CXCR4), β 1-integrin, CD34 and α 7-integrin⁸⁵. They do not express CD31, CD11b, CD45 and Sca1^{11,85}. Human satellite cells are positive for CD56, CD29 and epidermal growth factor receptor (EGFR). They don't express CD31, CD45 or mouse satellite cell marker CD34^{14,98}. Highly purified mouse and human satellite cell population can be obtained by FACS sorting and this protocol is well described^{11,14,85,99}. However, for most livestock species, the method to FACS purify satellite cells is not well established. Thus, it is necessary to develop a robust method to obtain and define highly purified satellite cells.

6. Maintaining the stemness of satellite cells

It has been reported that long term cultivation of mouse^{13,100}, dog¹⁰¹, or human¹⁴ satellite cells in vitro results in loss of most of the regenerative potential after transplantation back into mouse. The differentiation abilities in vitro also show defects after long-term culture¹⁰²⁻¹⁰⁴. Several intracellular signaling pathways have been implicated in maintaining stemness or conversely, move the cells towards a differentiated lineage or senescence.

6.1 p38 MAPK signal pathway

Further maintenance of satellite cell stemness can depend on cell signaling during proliferation. p38, a subgroup of the Mitogen-activated protein kinase (MAPKs), that can be activated by stress signals, inflammatory cytokines, and many other stimuli has been implicated in cell proliferation, senescence, apoptosis and other cellular processes^{105,106}. The p38 α / β MAPK signaling pathway regulates asymmetric division of satellite cells¹⁰⁷. One daughter cell activates p38 α / β MAPK, induces *MyoD* expression and generates a proliferating myoblast¹⁰⁷. In the other daughter cell p38 α / β MAPK signaling is not activated, *MyoD* is not induced, and the fate of the cell is directed towards a quiescent satellite cell that maintains the stem cell pool¹⁰⁷.

Previous studies have noted that p38-MAPK signaling plays an important role in the loss of stemness in satellite cells^{14,106,108,109}. Acute injury in p38 α -deficient mice resulted in a prolonged satellite cell response and an increased stem cell pool¹⁰⁹. Conversely, elevated activity of p38 α / β MAPK signaling induced regenerative defects in older satellite cells compared with younger ones¹⁰⁸. In the same model, inhibition of p38 α / β MAPK signaling and culture on soft hydrogel substrates rejuvenated older satellite cells' potential for regeneration¹⁰⁸. The p38-MAPK may play an important for long-term cultured myoblast cells.

6.2 JAK2-STAT3 signal pathway

Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway is known to have a critical role in cell growth, proliferation and differentiation in many cell types¹¹⁰⁻¹¹². In mammals, the JAK family kinases include four members (JAK1, JAK2, JAK3 and TYK2 (tyrosine kinase 2)), whereas the STAT family have seven (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6)^{113,114}.

The JAK-STAT pathway is not only important in regulating skeletal muscle mass, repair, aging and diseases in vivo¹¹⁴, but also can positively regulate the differentiation of satellite cells and myoblasts in vitro¹¹⁵. Following acute muscle damage, IL-6-induced activation of STAT3 signaling is essential for satellite cell activation and muscle regeneration^{116,117}. Two recent studies reported that JAK2-STAT3 signaling in muscle stem cells induce myogenic differentiation to the detriment of their expansion¹¹⁸⁻¹²⁰. Knockdown of STAT3 in satellite cells decreased the expression of both *MyoD* and *Myog*^{120,121}, thus demonstrating the pivotal role of STAT3 in satellite cell myogenic progression. In myoblasts, JAK1-STAT1-STAT3 plays a notable role in proliferation, whereas JAK2-STAT3 and JAK3 function mainly in differentiation^{112,115,122}. In summary, the JAK2-STAT3 signaling has double-edged effects in muscle regeneration and satellite cell function. Its role in long-term cultured myoblast still needs further research.

6.3 Loss of stemness is due to differentiation?

Several signals are up-regulated during satellite cell activation, such as p38 MAPK^{69,123,124} and JAK-STAT signals^{112,115,122}, which make the satellite cells generate more differentiated PAX7⁺MYOG⁺ daughter cells, aka myoblasts^{95,108,125}. The MYOG expression leads to the down-regulation of genes involved in cell cycle progression and promotes differentiation and fusion¹²⁶. While functional in tissue repair in vivo, this differentiation prevents further cell expansion in culture.

In the long-term passage conditions, the cells were cultured to less than 60% confluency and with enough nutrition. Even in those conditions there is a gradual loss of proliferation and differentiation abilities during long-term culture similar to the changes occurring during aging^{95,127}. Whether the differentiation impaired long-term cultured myoblast cells is discussed.

6.4 Stem cells function loss is due to stem cell aging?

Loss of stemness in muscle satellite cells is also related to aging and senescence. Aging is associated with a decline in the regenerative capacity of multiple tissues and organs in vivo^{128,129}. The number and the functionality of muscle stem cells decline with aging and this is accompanied by increased fibrosis and fatty degeneration, as a result of changes in niche function and intrinsic properties of SCs^{128,130,131}. Several intracellular processes and signaling pathways have been described as being dysregulated in association with ageing of satellite cells in vivo such as mitochondrial dysfunction^{132,133}, FGF2 and FGF

receptor 1 function⁹⁵, protein homeostasis (proteostasis)¹³⁴, p38^{95,108}, STAT3^{119,120}, p16¹³⁵, canonical Wnt¹³⁶ and Notch signaling¹³⁷, as well as epigenetic status^{128,138}.

The long-term culture induced limited replicative life-span of human fibroblast was firstly described in 1961 and was further designated as the replicative senescence¹³⁹⁻¹⁴¹. It is also called in vitro aging cells since replicative senescence showed similarities with in vivo aging¹²⁹. Long-term culture of satellite cells not only showed proliferation arrest^{13,14,103,127}, but also impaired differentiation ability^{134,102}. One of the features of cells subject to extensive subculturing is a gradual impairment of the glucose and lipid metabolism^{103,127}. However, more information on aging satellite cells in vitro is still needed^{103,127}.

Aims of this study

Cultured meat has been suggested to use approximately 7-45% lower energy use, 78-96% lower GHG emissions, 99% lower land use, and 82-96% lower water use than conventionally produced European meat according to the life cycle assessment (LCA) research¹⁴². This study mainly focuses on characterization and isolation of highly purified porcine and bovine satellite cells as cell sources for cultured meat. Since satellite cells lose proliferation and differentiation ability during in vitro culture, the reasons and solutions are also assessed.

1. To characterize satellite cells in porcine and cattle. Satellite cells have been well studied in human and mice for clinical usage. Less information is available in pig and bovine satellite cells. According to the information from mouse and human, pig and bovine satellite cells are characterized by transcription factors and surface markers that are reported as being important.
2. To isolate high purity satellite cells in porcine and bovine muscle by FACS. It has been a major focus to isolate a purified stem cell population to study stem cell biology. In the past few years, several groups have described the protocols to isolate satellite cells from the muscles tissue of mice and human by FACS. Using the identified surface markers, highly purified porcine and bovine satellite cells were isolated by FACS.
3. To monitor the satellite cells features during long-term culture. Large scale culture of functional satellite cells is a major issue in clinical field and cultured meat project. Loss of stemness has been found in mouse, dog and human satellite cells after culturing in vitro. Bovine and porcine satellite cells from different passages were collected and analyzed for stemness gene expression and differentiation ability.
4. To explore some differentiation and aging pathway effects in long-term cultured satellite cells. The differentiation process is accompanied by cell cycle withdrawal.

During aging, intrinsic and extrinsic stimuli drive functional decline of muscle stem cells. Several differentiation and aging related signal pathways were checked during long-term culture.

5. To systematic investigate the reason for function loss of long-term cultured satellite cells. The biological changes of long-term cultured satellite cells were analyzed by high throughput label-free quantitative proteomics.

6. To maintain the stemness of long-term cultured satellite cells. Both amount and function of satellite cells are important for clinical and cultured meat purposes. Several biological process and signal pathways were studied and intervened in. The approach to maintain the stemness and differentiation abilities by interfering those signals was evaluated.

Overview of the thesis

In **chapter 2** we cloned the full-length cDNA sequence of porcine PAX7 and characterized the numbers of satellite cells located in various pieces of skeletal muscle. The satellite cell frequency in variant pieces of porcine skeletal muscles were characterized. Then we established a FACS sorting method to isolate porcine satellite cells based on the surface markers. We further demonstrate that similar to satellite cells in other species, porcine satellite cells lose their stem cell identity and differentiation potential after long-term in vitro culturing.

Chapter 3 describes a fluorescence-activated cell sorting strategy to enrich bovine satellite cells. We find that p38-MAPK signaling is activated and PAX7 expression is gradually lost during satellite cell proliferation. The p38 α / β inhibitor SB203580 inhibits the differentiation of bovine satellite cells in short-term experiments while long-term cultivation with p38i helps maintain the stemness and differentiation abilities.

In **chapter 4** we show that the STAT3 signal was activated in cultured bovine satellite cells. The JAK2-STAT3 signal is required for bovine myoblast differentiation. Inhibition of JAK2-STAT3 signal impaired the differentiation process. However, JAK2-STAT3 inhibition does not help maintain the stemness gene, proliferation or long-term passage. Further proteomics and western blots results indicate that the JAK2-STAT3 signal was not the reason for the long-term culture defects in bovine satellite cells.

Chapter 5 describes several changes in signal pathways during differentiation and long-term culture process by Western blot. The two processes show similar up-regulation expression profiles in p38-MAPK signal. The JAK2-STAT3 signal pathway is up-regulated during differentiation but remains stable in long-term culture. Both ERK and Akt signal are down-regulated when the cells are confluent and then increased during

differentiation. However, ERK signal keeps decreasing while Akt signal increases during long-term culture.

In **chapter 6** we used proteomic analysis to identify the pathways increased and decreased during long-term passage. Biological pathway analysis shows that the proteins up-regulated in late passage are involved in cell-cell adhesion, muscle contraction, mitochondrial electron transport, NADH to ubiquinone, cell redox homeostasis. Early passages on the other hand show high expression of proteins involved in DNA replication, protein folding, translational initiation, DNA repair, fibroblast growth factor receptor signaling pathway, p53 regulation, proteasome- ubiquitin-dependent proteolysis, telomere maintenance via recombination and somatic stem cell population maintenance.

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Chapter 2

Characterization and isolation of highly purified porcine satellite cells

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Abstract

Pork is an important food source and the pig is an excellent animal model for human diseases. Careful characterization of the swine skeletal muscle stem cells (satellite cells) will shed a light on the generation of a swine skeletal muscle disease model and on efficient production of porcine meat for the food industry. PAX7 is a highly conserved transcription factor shared by satellite cells from various species. However, the sequence of *PAX7* has not been characterized in pig. The lack of a method to isolate highly purified satellite cells hinders the thorough characterization of swine satellite cells. Here we found molecular markers for swine satellite cells and revealed that porcine satellite cells were heterogeneous in various pieces of skeletal muscle. We further developed a method to isolate highly purified satellite cells directly from porcine muscles using fluorescence-activated cell sorting (FACS). We next characterized the proliferation and differentiation abilities of isolated satellite cells in vitro and found that long-term culturing of satellite cells in vitro led to loss of stemness.

Introduction

Satellite cells are a heterogeneous population of adult stem cells located in skeletal muscles^{1,2,3,4}. These cells reside between the muscle sarcolemma and the basal lamina of muscle fibers and remain quiescent under normal conditions^{1,2,5,6}. Upon injury, quiescent satellite cells are activated to repair muscle injury while a subset of the activated satellite cells return to quiescent status after self-renewal^{1,2,7}. Satellite cells are essential for muscle regeneration and mice lacking satellite cells display severe muscle regeneration defects^{8,9,10}. Satellite cell impairment occurs in muscular diseases such as Duchenne muscular dystrophy (DMD)¹¹ and in aged muscles¹².

Pigs have many advantages in modeling human diseases due to their similar anatomic and physiological features to human beings^{13,14}. For example, the swine DMD model recapitulates human symptoms better than the mouse model. The severe progressive dystrophic changes of skeletal muscles, impaired mobility, muscle weakness and a much shorter life span are common symptoms in human DMD patients¹⁵. These symptoms can only be recapitulated in the pig DMD model¹⁶, but not in the mouse DMD model¹⁷, supporting the notion that pigs can model human diseases better than rodents. Characterization of satellite cells in pig will be a valuable addition to our understanding of the porcine model system.

PAX7 has been shown to be the critical regulator of satellite cell maintenance and proliferation in several species^{18,19,20}. PAX7 is a highly conserved protein present in many mammalian species such as human, mouse, and cattle. In mice, PAX7 can directly regulate *MyoD* and *Myf5* to modulate satellite cell maintenance and proliferation^{21,22,23,24}. The full length CDS of *Pax7* has been found in the mouse (Accession #: NM_011039) and the human (Accession #: NM_001135254) database. For PAX7 in pig, however, there are currently only three short partial sequences in the database.

The frequency of satellite cells and their functions are heterogeneous in both mouse and human skeletal muscles^{4,25,26}. Mouse Soleus and diaphragm muscles have more satellite cells per mm³ compared to other muscles²⁵. In humans, the Temporalis muscle has estimated higher satellite cell numbers, whereas other body and limb muscles show lower satellite cell numbers²⁶. Little is known about the satellite cell frequency heterogeneity in pig muscles.

Highly purified mouse satellite cell populations can be obtained by FACS sorting but the method to purify porcine satellite cells is not well established. Several approaches have been reported to isolate the myogenic lineage cells from porcine muscle tissues, including Percoll gradient centrifugation and preplating^{27,28,29,30,31}. However, the cell identity of the myogenic lineage population obtained by the above methods has not been carefully characterized yet. The purification efficiency of Percoll gradient centrifugation shows big variation as reported by different groups^{27,32}. Furthermore, in cells obtained by Percoll gradient centrifugation, only a portion (<60%) of them stain

positive for neural cell adhesion molecule (N-Cam), which is considered to be a porcine satellite cell marker²⁸. Consistently, only 65% of the cells obtained by this method are able to fuse and form myotubes³³. Another widely used approach to isolate porcine myogenic lineage cells is the preplating method^{29,34,35}. PAX7 immunofluorescent staining indicates that satellite cells only account for 20%- 50% of the cell population obtained by this method^{29,35}. Thus, it is necessary to develop a more efficient method to obtain highly purified satellite cells.

Previous studies in mice have suggested that highly purified satellite cells can be obtained by FACS sorting^{2,36,37}. The cell surface markers for FACS sorting have been well characterized in murine muscle tissues. The most commonly used cell surface markers in murine are CD34 and α 7-integrin^{2,36,38}. The cell surface markers to specify pig satellite cells are not well characterized. It has been reported that CD56 was expressed in a myogenic cell population residing in human^{26,39} and swine skeletal muscles⁴⁰. Further analysis in human muscles showed that the cells expressing CD56 can be further divided into two populations based on CD34 expression. CD56⁺CD34⁻ cells isolated from skeletal muscles displayed only myogenic potency, whereas CD56⁺CD34⁺ cells showed both adipogenic and myogenic potentials⁴⁰, though the scenario in pigs has not been well characterized. CD29 (β 1-integrin) is a member of the integrin family that can form heterodimers to link the extracellular matrix to the actin cytoskeleton within cells⁴¹. CD29-deficient embryonic myoblasts displayed impaired fusion ability *in vitro*⁴². Satellite cells lacking CD29 fail to maintain a quiescent state and cannot sustain the expansion and self-renewal of this stem cell pool during regeneration⁴³. In mice, more than 95% of satellite cells are CD29 and PAX7 double positive⁴⁴; in human, 96% of satellite cells are CD29 and PAX7 double positive²⁶. These observations suggest that CD29 could be a surface marker to specify satellite cells together with other markers.

It has been reported that long-term cultivation of satellite cells *in vitro* resulted in loss of most of the regenerative potential *in vivo* after transplantation in mouse^{36,45}, canine⁴⁶, and human⁴⁷. Scattered reports have shown that the progenitor cells derived from swine skeletal muscles display multilineage differentiation capacities after being cultured *in vitro* for 3 month⁴⁸, but the identity of these cells has not been characterized. Whether the lack of ability to be expanded *in vitro* is also true in pig satellite cells is not known yet.

Here we characterize the satellite cell frequency in variant pieces of porcine skeletal muscles. The cell surface markers specific for porcine satellite cells have been identified, and a FACS sorting method to isolate porcine satellite cells based on the surface markers has been established. We further demonstrate that similar to satellite cells in other species, porcine satellite cells lose their stem cell identities and differentiation potentials after long-term *in vitro* culturing.

Results

Clone of the full length porcine *PAX7* gene

PAX7 is an important regulator of muscle satellite cells^{18,19,20}. We set out to compare the similarity of the *PAX7* peptide sequence between pig and other species. However, we could not find the full length *PAX7* sequence in the NCBI database. There are only several partial porcine *PAX7* cDNA sequences in NCBI nucleotide database (Accession #: AY653213, XM_013992407, XM_013991732). These translated peptide sequences were aligned with *PAX7* peptide sequences from several species (Figure 1a). Peptide sequence (Accession #: AAT72072) translated from AY653213 displayed 100% identity to *PAX7* peptide sequences from multiple species (Figure 1a and Supplementary Figure S1). *PAX3* belongs to the same family as *PAX7* and is very similar to *PAX7*. Sequence comparison indicated differences between two key amino acids in the peptide sequence derived from AY653213 and the *PAX3* amino acid sequence from various species (Supplementary Figure S1), suggesting that AY653213 could be a partial sequence of porcine *PAX7*.

To identify the complete cDNA sequence of *PAX7* in pig, we performed 5' and 3' rapid amplification of cDNA ends (RACE) using AY653213 as the seed sequence with total RNA extracted from porcine myoblasts (Supplementary Figure S2a). A 400bp fragment was generated by the 5' RACE; and a 5000bp fragment was generated by the 3' RACE (Supplementary Figure S2b). The 2 fragments could be assembled into a 1512bp CDS (Accession #: KX815346), which encoded a 503 amino acid polypeptide (Figure 1b). The assembled nucleic acid sequence showed 93% identity to human *PAX7* and 90% identity to mouse *PAX7* (Supplementary Figure S3a). The translated polypeptide displayed 99% identity to human *PAX7* and 98% identity to mouse *PAX7* protein (Supplementary Figure S3b). Paired box domain (*PAX*), homeobox domain (*Homeobox*) and Paired box protein 7 (*Pax7*) domain, the three highly conserved domains of *PAX7* from various species, were all found in the amino acid sequence translated from the full-length pig *PAX7* CDS (Figure 1b).

Figure 1. Cloning of the full length porcine PAX7 CDS

(a) Schematic representation of the PAX7 protein sequences from different species. Hatched segments indicate conserved domains. Mouse (*Mus musculus*, Ms), cattle (*Bos taurus*, Bt), chick (*Gallus gallus*, Gg) PAX7 proteins and partial pig (*Sus scrofa*, Ss) PAX7 peptide sequences were aligned with human (*Homo sapiens*, Hs) PAX7 protein. Black indicates the partial porcine PAX7 cDNA sequences. (b) Nucleotide sequence of pig PAX7 and the predicted protein sequence. The conserved Paired Box (PAX), Homeobox (Homeobox) and Paired box protein 7 (Pax7) are boxed.

The satellite cell number varied at different age and location

We tested several antibodies and found the antibody recognizing porcine PAX7 protein. This antibody was utilized to detect the endogenous porcine satellite cells. Consistent with observations in mouse¹⁸ and human²⁶, PAX7 positive satellite cells were detected underneath the basal membrane as indicated by Laminin immunofluorescent staining in both postnatal and adult pigs (Figure 2a and b).

The satellite cell number is important for skeletal muscle homeostasis maintenance. It has been reported that the satellite cell number decreased in adult when compared to neonatal mice⁴⁹. The number of satellite cells also differed between various pieces of skeletal muscles in the same individual²⁵. We next checked the satellite cell number in Semitendinosus from 1- and 25-week old pigs by PAX7 immunofluorescent staining. The number of satellite cells decreased in adult skeletal muscle (25-week old) compared to neonatal (1-week old) muscles in pig (Figure 2b and c). To compare the satellite cell numbers in different muscle pieces, we analyzed 9 types of adult pig skeletal muscle from different locations. Peroneus Tertius, Psoas Major, and Extensor carpi radialis had high numbers of satellite cells, whereas Intercostal muscle and Biceps femoris had low amounts of satellite cells (Figure 2d and e). These results suggest that the distribution of satellite cells in different types of skeletal muscle is different.

Identification the satellite cell specific cell surface markers

In order to set up a FACS sorting method to isolate porcine satellite cells, we next characterized the specificity of the surface markers for porcine satellite cells. CD56 and CD29 have been reported to mark satellite cells in human. We performed immunofluorescent staining assays to verify whether these surface markers were also applicable to porcine satellite cells. Both CD56 and CD29 co-localized in the same cell with PAX7 in porcine skeletal muscles (Figure 3a), suggesting that they could be used as cell surface marker for isolation of satellite cells from pig skeletal muscles. Antibodies against CD31 (an endothelial cell marker⁵⁰) and CD45 (a hematopoietic cell marker⁴⁸) were utilized to distinguish the endothelial and hematopoietic cells present in the mononuclear cells isolated from porcine skeletal muscles. Based on these results, we isolated CD31⁻CD45⁻CD56⁺CD29⁺ cells from mononuclear cell population obtained from porcine skeletal muscles by FACS sorting (Figure 3b). The *PAX7* and *MYF5* mRNA level in this cell population was compared with that in CD31⁺CD45⁺ and CD31⁻CD45⁻CD56⁻CD29⁺ populations by RT-qPCR. *PAX7* and *MYF5* are enriched in the CD31⁻CD45⁻CD56⁺CD29⁺ population (Figure 3c and Supplementary Figure S4a). Immunofluorescent staining assays revealed that 94% of CD31⁻CD45⁻CD56⁺CD29⁺ cells express PAX7 (Figure 3d, 3e and Supplementary Figure S4b), suggesting specific enrichment of PAX7⁺ cells in CD31⁻CD45⁻CD56⁺CD29⁺ cell population isolated from porcine skeletal muscles. Taken together, satellite cells can be isolated from pig skeletal muscles by FACS sorting using CD31 and CD45 negative selection followed by CD56 and CD29 positive selection.

Figure 2. The number of satellite cells varied at different age and location

(a) Immunofluorescent staining of the Semitendinosus muscle section from 25-week old pig using PAX7 and Laminin antibodies. Green: PAX7; Red: Laminin; Blue: DAPI. Scale bars: 20 μm . (b) Representative images of immunofluorescent staining of PAX7 (satellite cells) and Laminin on pig Semitendinosus cross sections obtained from 1-week or 25-week old pigs. Green: PAX7; Red: Laminin; Blue: DAPI. The green arrows indicate the satellite cells. Scale bars: 50 μm . (c) Quantifications of the satellite cell numbers in mice at different ages. Error bars represent S.E.M. and were based on 4 independent experiments. Significance was analyzed by student's t-test, *** indicates $P<0.001$. (d) Representative images of immunofluorescent staining of pig satellite cells on tissue sections derived from different muscle types in 25-week old pigs. Green: PAX7; Red: Laminin; Blue: DAPI. The green arrows indicate the satellite cells. Scale bars: 100 μm . (e) Quantifications of satellite cell number in different pieces of skeletal muscles. Error bars represent mean $\pm$ S.E.M. and are based on 4 independent experiments.

Figure 3. Identification the satellite cell specific cell surface markers

(a) Immunofluorescent staining of PAX7, CD56 (a, top), CD29 (a, bottom), and Laminin on cross sections of Semitendinosus muscle from 25-week old pig. Green: PAX7 (a, top) or CD29 (a, bottom); Purple: CD56 (a, top) or PAX7 (a, bottom); Blue: DAPI; Red: Laminin. Arrows indicate satellite cells expressing all three markers. Scale bars: 20 μ m. (b) FACS analysis of mononuclear cells from Semitendinosus obtained from 1-week old pig. Cells were gated by forward scatter and side scatter (not shown) prior to gating for CD45/31, CD56, and CD29. Red gates indicate sub-populations containing pig satellite cells. (c) qRT-PCR analysis of *PAX7* mRNA levels in freshly isolated pig satellite cells and other cell populations were shown. (d) Immunofluorescent staining of PAX7 in pig satellite cells cultured 4d in vitro. Blue: DAPI; Green: PAX7. Scale bars: 20 μ m. (e) Quantification of PAX7 immunofluorescent staining of sorted CD56⁺CD29⁺ cells and unsorted cells cultured 4d in vitro from 1-week old pig. Data represent mean $\pm$ S.E.M. and are based on 3 (figure 3e) or 4 (figure 3c) independent experiments. Significance was analyzed by student's t-test, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

To analyze the proliferation abilities of porcine satellite cells in vitro, we cultured the satellite cells for several days in vitro. After being cultured in vitro for 4 days, the cell size started to increase and the cell shape became more spindle-like (Figure 4a, b and c and Supplementary Figure S5a). Previous reports have shown that the cell size increased when satellite cells are being activated and start differentiating to myoblasts⁵¹. To further characterize the identity of the cells isolated from pig skeletal muscles by FACS sorting, we performed immunofluorescent staining of MyoD and PAX7. In the round satellite cells, which were cultured in vitro for less than 6h, PAX7 protein was detected and MyoD protein level was relatively low (Figure 4c). Cells being cultured for 4d were bigger, and PAX7 positive (Figure 4c). MyoD protein displayed punctuated distribution and was mostly located in nuclei (Figure 4c). The PAX7 mRNA level was decreased during in vitro culturing (Figure 4d). These results suggest that, consistent with the circumstance in mouse^{5, 51}, porcine satellite cells being cultured in vitro for over 4d were activated and started to differentiate to myoblasts.

We next assayed the differentiation potential of porcine satellite cells in vitro. Porcine satellite cells were differentiated in either 2% horse serum (HS) or 0.4% Ultrosor G (UG) (Figure 4e and 4f). The myosin heavy chain (*MyHC*) expression level was dramatically higher in UG-induced differentiation (Figure 4f). The differentiation efficiency was also enhanced by UG induction (Figure 4f).

We next examined whether cell density would affect pig satellite cell differentiation. We seeded 2×10^4 , 1×10^5 , and 5×10^5 pig satellite cells in a 3.5cm dish for 2 days (Supplementary Figure S5b) and induced differentiation with UG, respectively. When the density of the seeded pig satellite cells was low, the differentiation efficiency decreased; when the cell density reached the threshold, the differentiation efficiency plateaued (Figure 4g and h). Higher seeding cell density yielded myotubes with elevated MyHC expression levels and a higher percentage myotube nuclei (≥ 3 nuclei) of total nuclei (Figure 4i and j). Together, these results suggest that high cell density facilitates pig satellite cell differentiation. We further examined the contractility of the differentiated myotubes. After being cultured in medium containing UG and Ca^{2+} for 6 days, myotubes differentiated from swine satellite cells were able to contract. These results suggest that swine satellite cells were able to generate functional myotubes.

Figure 4. Proliferation and differentiation abilities of pig satellite cells *in vitro*

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(a) Representative phase contrast images of pig satellite cells cultured in F-10 medium for various hours. Scale bars: 10 μm . (b) Quantification of the cell size with different culturing time. (c) Representative immunofluorescent staining images of pig freshly isolated satellite cells or cultured in vitro for 96 h. In the inserts, Red: MyoD; Green: PAX7; Blue: DAPI. Scale bars: 10 μm . (d) qRT-PCR analysis of *PAX7* mRNA levels in freshly isolated pig satellite cells and satellite cells cultured for 4d. (e) Representative immunofluorescent staining images of MyHC of differentiated satellite cells at passage 1 with 0.4% UG and 2% HS. Green: MyHC; Blue: DAPI. Scale bars: 50 μm . (f) qRT-PCR analysis of *MyHC* mRNA levels with cells differentiated in 0.4% UG and 2% HS conditions (f, left). Quantification of the number of MyHC⁺ cell numbers with cells differentiated in 0.4% UG and 2% HS conditions (f, right). (g) Representative immunofluorescent staining images of MyHC with different amount of seed cells at passage 3. Cells were cultured with 2×10^4 (left), 1×10^5 (middle), 5×10^5 (right) cells for 2 days, then induced to differentiate in 0.4% UG. Green: MyHC; Blue: DAPI. Scale bars: 50 μm . (h) Quantification of the number of MyHC⁺ cells in cells differentiated from different amount of seed cells, (i) qRT-PCR analysis of *MyHC* mRNA levels in cells differentiated from different amount of seed cells. (j) Quantification of the percentage of nuclei in myotubes differentiated from different amount of seed cells. Bars represent mean $\pm$ S.E.M. and are based on 3 independent experiments. Significance was analyzed by student's t-test or one-way ANOVA. * indicates $p < 0.05$, ** indicates $p < 0.01$. *** indicates $p < 0.001$. NS: Non-significant.

Porcine satellite cells lost their stemness after long-term in vitro expansion

Both mouse and human satellite cells have been reported to suffer loss of their ability to repair muscle injury in vivo after days of in vitro culturing^{5,36,45,47,52}. We therefore examined whether porcine satellite cells have the same feature. As described above, the cell size of porcine satellite cells increased and the cell shape became more spindle-like after 4d of in vitro culturing (Figure 4a), suggesting these cells to be in a more differentiated status. The morphology of porcine satellite cells remained to be spindle-like after serial expansion in vitro (Figure 5a). These cells could be propagated logarithmically in vitro (Figure 5b). To further characterize the identity of the cells being serially expanded in vitro, CD56 and CD29 expression was analyzed by FACS. The expression level of both CD56 and CD29 decreased after passage 4 (Figure 5c), suggesting a gradual loss of satellite cell character. Consistently, the percentage of PAX7⁺ cells also decreased dramatically after passage 4 and 5 (Figure 4d). The expression level of PAX7 echoed the morphology changes observed above. After 4d of in vitro culturing, the PAX7 mRNA level dropped more than 30-fold (Figure 5e). Serial expansion in vitro of these cells further drove the PAX7 mRNA level and the percentage of PAX7⁺ cells down (Figure 5d and e). After passage 4 and 5, the PAX7 expression level decreased to a very low level (Figure 5e), suggesting that these cells had lost their satellite cell features. Consistently, the cells expanded in vitro for over 5 passages displayed significant lower differentiation potentials (Figure 5f and g). Taken together, these results suggest that pig satellite cells quickly lose their stem cell features in vitro.

Figure 5. Porcine satellite cells lost their stemness after long term expansion in vitro

(a) Representative phase contrast images of pig satellite cells cultured in F10 medium for different passages. Scale bars: 200 μ m. (b) Growth curves of pig satellite cells isolated from 1-week old pig. Cells were serially expanded and counted at each passage. (c) FACS analysis of CD56 and CD29 expression from different passages of pig satellite cells cultured in F-10 medium. (d) Quantification of the number of PAX7⁺ cells from different passages. (e) qRT-PCR analysis of PAX7 mRNA levels from cells being expanded for different passages. (f) Representative immunofluorescent staining images of MyHC staining of myotubes differentiated from P0, P2, P5 and P10 satellite cells. Green: MyHC; Blue: DAPI. Scale bars: 50 μ m. (g) Quantification of MyHC⁺ cell numbers differentiated from satellite cells being serially expanded in vitro. Bars represent mean $\pm$ S.E.M. and were based on 3 independent experiments. Significance was analyzed by student's t-test or one-way ANOVA. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$. NS indicates no significant difference.

We further examined the ability of pig satellite cells to repair muscle injury in vivo by cell transplantation. Satellite cells were isolated from 1-week old piglets by FACS sorting. Tibialis Anterior (TA) muscle injury in immunodeficient recipient mice (NOD.*Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ*) was induced by cardiotoxin injection. Various numbers of pig satellite cells were transplanted to the recipient mice. Four weeks after transplantation, TA muscles from recipient mice were harvested to make cryo-sections and stained with antibody specifically recognizing pig LAMIN A/C. Pig LAMIN A/C could be detected in TA muscles of the recipient mice (Figure 6a and Supplementary Figure S6a). One week after transplantation, the porcine LAMIN A/C could also be detected in newly formed myofibers (indicated by MYH3 staining) (Figure 6b), suggesting that the transplanted pig satellite cells were able to engraft and contribute to muscle regeneration in recipient mice. We further examined whether the transplanted swine satellite cells were capable of homing to the right niche. PAX7 expressing satellite cells were detected in TA muscles of the recipient mice four weeks after transplantation (Supplementary Figure S6b). The PAX7 expressing satellite cells of pig origin (indicated by LAMIN A/C staining) were detected at the similar location where the endogenous satellite cells resided (Supplementary Figure S6c). These results suggest that the transplanted swine satellite cells were capable of homing to the niche.

We next investigated whether in vitro expansion of porcine satellite cells affects their engraftment efficiency. The same number of freshly isolated pig satellite cells and porcine satellite cells expanded in vitro for different periods were transplanted to immunodeficient recipient mice, respectively. The antibody specifically recognizing porcine LAMIN A/C was used to perform immunofluorescent staining with muscle tissue sections 4 weeks after transplantation. The pig satellite cells expanded in vitro for 2 passages displayed similar engraftment efficiency as the freshly isolated pig satellite cells. However, few myofibers generated from pig satellite cells could be detected when these cells were expanded in vitro for 5 passages (Figure 6c and d), suggesting that pig satellite cells lost their stemness and ability to repair muscle injury in vivo after prolonged in vitro expansion. These results were also consistent with the morphology and differentiation potential changes described above (Figure 4 and 5), suggesting that pig satellite cells lost their stem cell features after being expanded in vitro for 5 passages. Taken together, these results suggest that pig satellite cells lose their stemness and differentiation abilities after in vitro expansion, a feature shared by both mouse and human satellite cells.

Figure 6. Pig satellite cells participated in the muscle regeneration in recipient mice and lose the engraftment efficiency after long-term expansion in vitro

(a) Four weeks after transplantation, engraftment efficiency of freshly isolated pig satellite cells was determined by DYSTROPHIN and LAMIN A/C immunofluorescent staining with muscle sections derived from recipient mice. PBS was injected as negative control. Blue: DAPI; Red: LAMIN A/C; Green: DYSTROPHIN. Scale bars: 20 μ m. (b) LAMIN A/C, MYH3 immunofluorescent staining with series muscle sections derived from recipient mice 7 days after transplantation. Blue indicated DAPI; red indicated LAMIN A/C (b, left) or MYH3 (b, right). Green: DYSTROPHIN. Scale bars: 100 μ m. (c) Representative immunofluorescent staining images of DYSTROPHIN and LAMIN A/C on mouse sections from mice injected with freshly isolated (c, left), P2 (c, middle) and P5 (c, right) satellite cells. Blue: DAPI; Red: LAMIN A/C; Green: DYSTROPHIN. Scale bars: 100 μ m. (d) Quantification of the number of LAMIN A/C⁺ fibers in each TA 4 weeks after transplantation. Significance was analyzed by one-way ANOVA. * indicates $p < 0.05$, ** indicates $p < 0.01$. NS indicates no significant difference.

Discussion

Here we characterized pig muscle satellite cells and provided basic information for further utilization of these cells. We cloned the full-length cDNA sequence of porcine PAX7 and characterized the numbers of satellite cells located at various piece of skeletal muscles. A FACS sorting based method was established to isolate highly purified pig satellite cells. Using this method, we isolated pig satellite cells and found that they lost their stem cell morphology, differentiation potential, and ability to repair muscle injury in vivo after prolonged in vitro culturing.

Neonatal mice have high numbers of satellite cells, while the number decreased in the adult (reviewed by Fu X et al⁵³). A similar phenomenon has been observed in pigs. The number of satellite cells was much higher in 1-week old pigs compared to that in 25-week old pig. The number of satellite cells varied at different locations. In mouse, extraocular and diaphragm have high number of satellite cells, Gastrocnemius and Tibialis Anterior have low number of satellite cells²⁵. In human muscles, it is indicated that under normal homeostatic conditions, most adult human skeletal muscles contain a relatively homogeneous frequency of PAX7 satellite cells per fiber²⁶. Since pig and human have larger fibers than mouse, whether pig fibers contain the relatively constant frequency of satellite cells still needs more studies.

The loss of ability to repair muscle injury in vivo of satellite cells after being cultured in vitro (empty amplification) is a common problem shared by mouse and human satellite cells^{5,36,47,52}. This problem hampers the application of satellite cell in the clinic. Our results revealed that pig satellite cells have the same problem. It suggests that the factors required for stemness maintenance are lacking in the in vitro culturing system. Recently, Fu et al found that cytokines secreted by activated T cells during the acute inflammation after muscle injury can help maintain the stemness of mouse satellite cells in vitro for over 2 months³⁶. Charville et al. showed that inhibition of p38 signaling is important to maintain the quiescent stage and stemness of human satellite cells⁴⁷. Whether these factors also work to maintain stemness of pig satellite cells is an interesting question to pursue.

Materials and Methods

Animals and pig muscle tissues

All animal procedures were performed according to institutional and national guidelines and approved by the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences in this study. Pig muscle tissues were collected from commercial pig “large white” killed at euthanized (asphyxiated by CO₂ of 1 week old, or stunned electrically and then killed by cutting the jugular vein of 25-week-old). The NOD.Cg-*Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ* (NSG) mice were purchased from Jackson Laboratories.

Pig satellite cells isolation

Pig satellite cells were isolated from 1 or 25 week old male pigs as previously described and adapted to pig tissues^{26,47}. Briefly, freshly harvested pig muscle was either immediately digested or kept in DMEM (Gibco) at 4°C. The tissues were dissected and dissociated with collagenase D (Roche 2mg/ml) and dispase II (Roche 1.07U/ml) in DMEM supplement with 1% penicillin-streptomycin (P.S.) at 37°C for 1.5 h. The mixture was triturated with pipette once per 15-20 min. After digestion, the muscle was aspirated and ejected in and out of the syringe 10 times with a 20-ml syringe and a 20-gauge needle. After centrifuging at 100 g for 5 min, the supernatant was collected and centrifuged at 1000g for 5 min at 4°C. The cells were washed with 20% FBS in DMEM and filtered through a 100 µm cell strainer followed by a 40 µm cell strainer. The cells were then centrifuged at 1000g for 5 min at 4°C and incubated with the erythrocyte lysis buffer (ACK) buffer for 5 min on ice. Then the cells were washed with PBS twice and cell pellet was reconstituted with FACS buffer (1% BSA in PBS) or frozen in FBS supplement with 10% dimethyl sulfoxide (DMSO).

For freezing cells, the cells were recovered in 37°C water bath and washed with PBS twice. The cells were reconstituted with FACS buffer and stained with an antibody cocktail consisting Alexa Fluor 647 anti-pig CD45 (1:20 BIO-RAD Cat# MCA1222A647), APC-conjugated anti-pig CD31 (1:20 BIO-RAD Cat# MCA1746APC), Alexa Fluor 488 anti-human CD29 (1:40 BioLegend, Cat#303016), PE-conjugated anti-human CD56 (1:40 BioLegend, Cat# 304606) for 30-45min on ice. After antibody incubation, the cells were washed with cold PBS for two times and reconstituted in F-10 with 20% FBS. The viable CD31⁺CD45⁺CD56⁺CD29⁺ cells were isolated. Also, the CD31⁺CD45⁺ cells and CD31⁺CD45⁺CD29⁺CD56⁺ cells were also isolated to extract RNA. Cell sorting was performed with a BD Influx cell sorter using 488nm, 561nm, and 640nm lasers. Unstained cells were routinely used to define FACS gating parameters.

Satellite cell culture and differentiation

Dishes (Corning) were coated with 0.05% collagen type I (Corning). FACS isolated pig satellite cells were cultured on collagen-coated dishes in F10 medium (F10 medium (Gibco) containing 15% FBS, 5 ng/ml FGF (Invitrogen) and 1% P.S.). Freshly isolated

satellite cells were collected and plated in collagen coated dish and cultured for less than 6 h to attach. Cells cultured at various hours were fixed with 4% paraformaldehyde (PFA) for immunofluorescent staining. For serial expansion, Cells were serially passaged to maintain a density of <60% confluence and counted at each passage. Pig satellite cells differentiation were induced with DMEM (Invitrogen) with 2% horse serum (Hyclone) or 0.4% Ultrosor G (Pall Corporation 15950-017). Differentiation was induced for 4 days. The expanded and differentiated cells were fixed with 4% PFA for immunofluorescent staining.

To observe the myotube contraction, pig satellite cells were differentiated in DMEM containing 0.4% Ultrosor G (Pall Corporation 15950-017) for 6 days, medium was changed at day 4.

For preplating method, isolated mononuclear cells were firstly cultured in F10 basal medium for 2 days. Cells were then treated with 0.25% trypsin for 5 min and stopped with F10 basal medium, centrifuged and re-plated. To separate satellite cells from fibroblast cells, the cells were incubated in an uncoated dish at 37 °C for 60 min and then transferred to a new collagen-coated dish. This preplate technical was repeated 3 times to remove residual fibroblast cells. The bright field images were acquired by Olympus DP73 microscope.

Immunofluorescent analysis of cultured cells

Cultured satellite cells were fixed with ice-cold 4% PFA (in PBS) for 20 min, rinsed with PBS, and permeabilized in 0.5% Triton X-100 (in PBS) for 8 min. Fixed and permeabilized cells were blocked and incubated with primary antibodies 1% BSA (in PBS) overnight at 4°C. Primary antibodies recognizing PAX7 (1:100, Developmental Studies Hybridoma Bank, Cat# PAX7), MyoD1 (1:200, abclonal, Cat# A0671), Myosin heavy chain (1:1000, Upstate, Cat# 05-716). After washing with PBS, cells were incubated with secondary antibodies for 1 h at room temperature. Next, the cells were stained with Alexa 488-, 561- labeled anti-mouse or -rabbit antibodies (Invitrogen) and then mounted with VECTASHIELD mounting medium with DAPI (Vector Laboratories Cat# H-1200). All images were acquired by Leica SP8 confocal microscope and processed with Adobe Photoshop CS5 to adjust brightness and contrast for publication.

For the FACS analysis of cultured satellite cells, about 10^5 cells were collected at each passage. The cells were fixed by 2% PFA for 15 min and washed 5 min 3 times. The cells were then incubated with Alexa Fluor 488 anti-human CD29 (1:40 BioLegend, Cat#303016), PE-conjugated anti-human CD56 (1:40 BioLegend, Cat# 304606) in 1% BSA in PBS for 30-45 min. After antibody incubation, the cells were washed with PBS for two times and reconstituted in PBS. FACS analysis was performed in LSR II (BD Biosciences) cell analyzer. Unstained cells were routinely used to define FACS gating parameters.

Pig muscle analysis

The pig muscle tissues were cut into small pieces and then frozen in liquid nitrogen. Cross sections were cut at 8 μm and processed for immunofluorescent staining. The cross sections were fixed with 4% PFA at room temperature for 10 min, washed in PBS. The sections were permeabilized with 0.5% Triton X-100 for 6 min and washed with PBS again. Sections were then blocked for 1 h with 10% goat serum at room temperature. For PAX7 and Laminin co-staining, sections were incubated at 4°C overnight with the following primary antibodies: anti-Laminin antibody (1:50, Sigma, Cat# L9393) or rat anti-Laminin (1:1000, Abcam, Cat# 11576), mouse anti-PAX7 (1:25, Developmental Studies Hybridoma Bank, Cat# PAX7). After PBS wash for 5 min 3 times, the sections were stained with Alexa 488-, 561- or 647-labeled anti-mouse or -rabbit or-rat antibodies (Invitrogen) and then mounted with VECTASHIELD mounting medium with DAPI (Vector Laboratories Cat# H-1200).

For sections co-stained with PAX7, Laminin and CD56/CD29, PAX7 and Laminin co-staining were firstly performed. After incubation with the secondary antibody above, sections were washed with PBS for 5 min 3 times and incubated with the Alexa Fluor 488 anti-human CD29 (1:25, BioLegend, Cat# 303016), APC anti-human CD56 (1:25, BioLegend, Cat# 304610) for 2-4h at room temperature. After washing with PBS for 3 times, sections were mounted with VECTASHIELD mounting medium with DAPI (Vector Laboratories Cat# H-1200). All images were acquired by Leica SP8 confocal microscope and processed with Adobe Photoshop CS5 to adjust brightness and contrast for publication.

Pig satellite cells transplantation

NSG mice were pretreated with 18 gamma (Gy) 1 days before transplantation limited to the hindlimb. Muscle injury was induced by the injection of CTX (Sigma) to TA muscle. Briefly, 15 μl of 10 μM CTX was injected at each TA muscle using 28-gauge needles. After 24h, freshly isolated and cultured pig satellite cells (treated by trypsin) at passage 2 and 5 were counted, centrifuged, and resuspended in 15 μl PBS with 10^5 cells. Cells were injected into the TA muscle using 28-gauge needles. Recipient mice were treated with daily drinking water supplement with neomycin sulfate (0.25mg/mg) for 2 weeks. The transplanted TA muscles were harvested 1 week, 4 weeks after transplantation in liquid nitrogen. Serial 10 μm transverse sections of the TA muscle were stored in -80°C fridge.

All glass slides were removed from -80°C and warmed at room temperature for 10 min. Sections were rehydrated with PBS and fixed in 4% PFA for 10 min at room temperature. After washed with PBS for 5 min 3 times, the sections were permeabilized with 0.5% Triton X-100 for 6 min and again washed with PBS. Then Sections were blocked for 1 h in 10% goat serum (in PBS) with mouse-on-mouse blocking reagent (Vector Laboratories). After blocking, sections were washed with PBS and incubated overnight with primary antibodies at 4°C. Next day, sections were then washed with PBS for 5 min 3 times

and incubated with secondary antibodies 1h at room temperature. Finally, sections were washed with PBS and mounted with VECTASHIELD mounting medium with DAPI (H-1200 Vector Laboratories, Burlingame, CA). Antibodies used in the staining were mouse anti-PAX7 (1:20, Developmental Studies Hybridoma Bank, Cat# PAX7), mouse anti-MYH3 (1:100, Developmental Studies Hybridoma Bank), mouse anti-LAMIN A/C (1:50, Santa Cruz Biotechnology, Cat# sc-7292), rabbit anti-DYSTROPHIN antibody (1:50, abcam, Cat# ab15277). The sections were next stained with Alexa 488-, 561- labeled anti-mouse or -rabbit antibodies (Invitrogen). All images were acquired by Leica SP8 confocal microscope and processed with Adobe Photoshop CS5 to adjust brightness and contrast for publication.

Gene expression analysis

RNA was extracted from cells using the RNeasy pure cell/bacteria kit including DNase treatment (QIAGEN) according to the manufacturer's instruction. One µg of total RNA from each sample was reverse transcribed to cDNA using M-MLV transcriptase (Promega) according to the manufacturer's instruction. Relative gene expression was determined was performed in triplicate using a SYBR Green PCR master mix on an ABI 7500 Fast Real-Time PCR system.

The primers used in these assays were the followings:

PAX7-F, 5'- GGTGGGGTcTTCATCAATGG -3',
PAX7-R, 5'- GTCTCTTGGTAGCGGCAGAG -3',
MYF5-F, 5'- AGAAGGTCAACCAGGCGTTT -3',
MYF5-R, 5'- GTAGCGGATGGCATTCTGA -3',
GAPDH-F, 5'- CAAGGAGTAAGAGCCCCTGG -3',
GAPDH-R, 5'- AGTCAGGAGATGCTCGGTGT -3'.

5' and 3' rapid amplification of cDNA ends

RNA was isolated from primary pig myoblasts by preplate method. The original sequence was based on the published partial pig *PAX7* cDNA sequence (Accession #: AY653213). We designed 4 pairs of gene specific primers: P1, P2, P3, P4 for 5'-RACE, P5, P6, P7, P8 for 3'-RACE. RACE experiments were performed with SMARTer RACE 5'/3' Kit (Clontech) following the manufacturers' protocols. Finally, we cloned the result cDNA into the pUC19-based vector and sequenced. The peptide and nucleotide sequences were aligned by the NCBI online Basic Local Alignment Search Tool.

The primers used were:

P1, 5'-GGTGGGGTTTTCATCAATGGGCGACC -3',
P2, 5'-CCACATCCGCCACAAGATAGTAGAG -3',
P3, 5'-TGCCCAACCACATCCGCCACAAGATAG -3',
P4, 5'-AGTAGAGATGGCCACACGGCATCCG -3',
P5, 5'-GTCTCTTGGTAGCGGCAGAGGAT -3',

P6, 5'-AGGCCGGATGGACCCGGTCTCTTGG -3',
 P7, 5'-TGTCTGGGCTTGCTGCCTCCAATGG -3',
 P8, 5'-TCCACATCCGAGTCGCCACCTGTCTG -3'.

2

Cell size, PAX7⁺ cells population, differentiation and engraftment efficiency measurement

The cell sizes were measured by Image J software (NIH). Bright field images of satellite cells cultured at different time points were acquired using Olympus DP73 microscope. Immediately after isolation, freshly isolated satellite cells were firstly plated and allowed to adhere for less than 6h at 37 °C.

PAX7⁺ cells population was expressed as the number of nuclei co-stained with DAPI and PAX7 divided by the total number of nuclei in the same field. More than 400 nuclei from 5 randomly chosen fields were analyzed.

MyHC⁺ cells population was expressed as the number of nuclei co-stained with MyHC⁺ and DAPI divided by the total number of nuclei in the same field. More than 3000 nuclei from 4 randomly chosen fields were analyzed.

The percentage of myotube nuclei in total nuclei was expressed as the nuclei inside myotube (at least 3 nuclei) divided by total nuclei in the same field. More than 3000 nuclei from 4 randomly chosen fields were analyzed.

Statistics

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software). For comparisons of two treatment groups, a Student's t-test was used. For more than two groups, one-way ANOVA was used. Results are means ± S.E.M. unless stated. P < 0.05 is considered significant.

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Supplementary information

Figure S1. Comparison of the partial peptide sequences of PAX3 and PAX7 in cattle, human, mouse and pig. Related to Figure 1

PAX3 (Bt)	1	GGVFINGRPLPNHIRHKIVEMAHHGIRPCVISRQLRVSHGCV	42
PAX3 (Hs)	1	GGVFINGRPLPNHIRHKIVEMAHHGIRPCVISRQLRVSHGCV	42
PAX3 (Ms)	1	GGVFINGRPLPNHIRHKIVEMAHHGIRPCVISRQLRVSHGCV	42
PAX3 (Ss)	1	GGVFINGRPLPNHIRHKIVEMAHHGIRPCVISRQLRVSHGCV	42
PAX7 (Bt)	1	GGVFINGRPLPNHIRHKIVEMAHHGIRPCVISRQLRVSHGCV	42
PAX7 (Hs)	1	GGVFINGRPLPNHIRHKIVEMAHHGIRPCVISRQLRVSHGCV	42
PAX7 (Ms)	1	GGVFINGRPLPNHIRHKIVEMAHHGIRPCVISRQLRVSHGCV	42
PAX7 (Ss: AAT72072)	1	GGVFINGRPLPNHIRHKIVEMAHHGIRPCVISRQLRVSHGCV	42
Consensus	1	GGVFINGRPLPNHIRHKIVEMAHHGIRPCVISRQLRVSHGCV	42
PAX3 (Bt)	43	SKILCRYQETGSIRPGAIGGSKPKQVITPDVEKK	76
PAX3 (Hs)	43	SKILCRYQETGSIRPGAIGGSKPK-VITPDVEKK	76
PAX3 (Ms)	43	SKILCRYQETGSIRPGAIGGSKPKQVITPDVEKK	76
PAX3 (Ss)	43	SKILCRYQETGSIRPGAIGGSKPKQVITPDVEKK	76
PAX7 (Bt)	43	SKILCRYQETGSIRPGAIGGSKPR-VATPDVEKK	76
PAX7 (Hs)	43	SKILCRYQETGSIRPGAIGGSKPRQVATPDVEKK	76
PAX7 (Ms)	43	SKILCRYQETGSIRPGAIGGSKPRQVATPDVEKK	76
PAX7 (Ss: AAT72072)	43	SKILCRYQETGSIRPGAIGGSKPRQVATPDVEKK	76
Consensus	43	SKILCRYQETGSIRPGAIGGSKP QV TPDVEKK	76

The comparison of partial predicted peptide sequences of PAX3 and PAX7 in cattle, human, mouse and pig. The green and blue background indicates differences on two key amino acids between PAX3 and PAX7 peptide sequence.

Figure S2. Identification of the completed cDNA sequence of pig *PAX7* by RACE. Related to Figure 1

(a) The strategy to generate full-length pig *PAX7* cDNA sequence by RACE. The red band indicates the seed sequence for RACE. (b) The 5' and 3' cDNA fragments generated by 5' and 3' RACE reactions. GSP indicates gene specific primer, NGSP indicates nested gene specific primer. The band generated by 5' RACE was about 400bp (b left) and a 5kb fragment was generated by the 3'RACE (b right).

Figure S3. Alignment of pig PAX7 polypeptide sequence from different species. Related to Figure 1

(a) Alignment of the full-length pig PAX7 CDS with human and mouse Pax7. Hatched indicates the conserved domains. Human (*Homo sapiens*, Hs) and mouse (*Mus musculus*, Ms) PAX7 cDNA sequences were aligned with assembled pig PAX7 cDNA sequence. (b) The human and mouse PAX7 polypeptide sequences were aligned with a pig PAX7 polypeptide sequence derived from full length CDS.

Figure S4. Identification of the cell surface marker for swine satellite cells. Related to Figure 3

(a) RT-qPCR analysis of *MYF5* mRNA levels in freshly isolated pig satellite cells and other cell populations. Error bars represented S.E.M. and were based on 4 independent experiments. Significance was analyzed by student's t-test, *** indicates $p < 0.001$. (b) Immunofluorescent staining of PAX7 in pig satellite cells cultured 4 days in vitro. Cells were stained with antibodies against PAX7 and with DAPI to identify nuclei. Blue: DAPI; Green: PAX7. Scale bars: 50 μm .

Figure S5. Related to Figure 4. *In vitro* proliferation and differentiation features of pig satellite cells. Related to Figure 4

(a) Representative phase contrast images of pig satellite cells cultured in F-10 medium for various hours. Scale bars: 100 μ m. (b) Representative phase-contrast pictures of 2×10^4 , 1×10^5 , 5×10^5 cells cultured in F10 medium in 3.5cm dish for 2 days. Scale bars: 200 μ m.

Figure S6. Swine satellite cells contributed to muscle regeneration in recipient mice. Related to Figure 6

(a) Four weeks after transplantation, engraftment efficiency of freshly isolated pig satellite cells was determined by DYSTROPHIN and LAMIN A/C immunofluorescent staining with muscle sections derived from recipient mice. PBS was injected as negative control. Blue: DAPI; Red: LAMIN A/C; Green: DYSTROPHIN. Scale bars: 100µm. (b) PAX7, immunofluorescent staining with muscle sections derived from recipient mice four weeks after transplantation. Blue: DAPI; Red: PAX7; Purple: DYSTROPHIN. Arrow and arrowhead indicate PAX7 expressing satellite cells. Scale bars: 100µm. (c) Pax7, LAMIN A/C immunofluorescent staining with series muscle sections derived from recipient mice four weeks after transplantation. Blue: DAPI; Red: PAX7 (c, top) or LAMIN A/C (c, bottom); Purple: DYSTROPHIN. Scale bars: 20µm.

Chapter 3

Maintaining bovine satellite cells stemness through p38 pathway

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Abstract

Isolating and maintaining the appropriate stem cell for large scale cell culture is essential in tissue engineering or food production. For bovine satellite cells an optimized isolation and purification protocol is lacking and there is also no detailed understanding on the factors that maintain stemness of these cells. Here, we set up a fluorescence-activated cell sorting strategy to enrich bovine satellite cells. We found that p38-MAPK signaling is activated and PAX7 expression is gradually lost during satellite cell proliferation. The p38 inhibitor (SB203580) treatment maintained *PAX7* expression but inhibited the fusion of satellite cells in a concentration-dependent way in short-term incubation. The mechanism of p38 inhibition was confirmed by inhibiting canonical p38 signaling, i.e. HSP27. Long-term culture with an appropriate concentration of p38i enhanced the proliferation and *PAX7* expression, while the differentiation capacity recovered and was enhanced compared to vehicle control. These studies indicate that bovine satellite cells maintenance depends on cell purity and p38 MAPK signaling. Inhibition of p38 MAPK signaling is a promising strategy to facilitate large scale cell expansion of primary cells for tissue engineering and cultured meat purposes.

Introduction

Satellite cells, initially identified by Mauro¹ in 1961, are the bona fide muscle stem cells. These cells are located beneath the sarcolemma and the basal membrane and originate from the dermomyotome cell population². During peri- and postnatal development, satellite cells contribute new nuclei to growing muscle fibers by fusing with the adjacent fiber^{3,4}. Subsequently, they enter a quiescent stage and are activated in injured muscle or for further muscle growth^{3,5,6}. Understanding the biology of satellite cells will help understand skeletal muscle regeneration, ageing, disease⁵ as well as the emerging field of culturing meat. Culturing meat for consumption uses stem cells to culture muscle tissue for future meat consumption with potential benefits for the environment, animal welfare and food security^{7,8}. This technology depends heavily on the ability of satellite cells to expand to high numbers of cells, for instance by maintaining their stemness while providing fast-growing myoblast colonies^{7,9}.

Maintaining or improving stemness, requires a highly purified satellite cell population. Bovine satellite cells are usually isolated by the preplating method^{10,11}, which in the absence of further purification leads to a purity of 31% based on fusion index or 95% by DESMIN staining^{11,12}. Satellite cells can be further purified using cell surface markers, but these are mostly characterized for mice and humans¹³⁻¹⁵ as well as for pigs¹⁶, not for cattle. Highly purified mouse¹⁷, human^{15,18} and pig¹⁶ satellite cell populations can be obtained by fluorescence-activated cell sorting (FACS) using CD34, β 7-integrin, CD56 or CD29^{15-17,19}. Expression of these markers is species specific. For instance, mouse satellite cells express CD34, whereas human satellite cells do not^{17,18,20}. Bovine satellite cell marker expression is not well characterized. We therefore first set out to purify the initial population of bovine satellite cells, based on marker expression.

Further maintenance of satellite cell stemness can depend on cell signaling during proliferation. p38, a subgroup of the Mitogen Activated Protein Kinases (MAPKs), can be activated by stress signals, inflammatory cytokines, and many other stimuli and has been implicated in cell proliferation, senescence, apoptosis and other cellular processes^{21,22}. The p38 α / β MAPK signaling pathway regulates asymmetric division of satellite cells²³. One daughter cell activates p38 α / β MAPK, induces *MyoD* expression and generates a proliferating myoblast²³. In the other daughter cell p38 α / β MAPK signaling is not activated and *MyoD* is not induced, thus renewing the quiescent satellite cell to maintain the stem cell pool²³.

Previous studies have noted that p38-MAPK signaling plays an important role in the loss of stemness in satellite cells^{18,22,24,25}. Acute injury in p38 α -deficient mice resulted in a prolonged satellite cell response and an increased stem cell pool²⁵. Conversely, elevated activity of p38 α / β MAPK signaling induced regenerative defects in older satellite cells compared with younger ones²⁴. In the same model, inhibition of p38 α / β MAPK signaling and culture on soft hydrogel substrates rejuvenated older satellite cells' potential for regeneration²⁴.

In a purified bovine satellite cell population, we investigated if bovine satellite cells showed up-regulated p38 MAPK signaling accompanied by a loss of differentiation ability during long-term culturing in vitro and if p38 inhibition could rescue stemness of satellite cells. Specifically, we found that the p38 α/β inhibitor SB203580 inhibited the differentiation of bovine satellite cells in short-term experiments while long-term cultivation with p38i helped maintain the stemness and differentiation abilities.

Results

FACS purification of bovine satellite cells

To isolate bovine satellite cells by FACS method, we firstly analyzed PAX7, CD56 and CD29 protein expression in mature bovine muscle fibers (Figure 1a). PAX7 is the most specific marker of satellite cells^{26,27} and bovine satellite cells were recognized by PAX7 nuclear staining. Both CD56 and CD29 co-stained with PAX7 in bovine skeletal muscle fibers (Figure 1a). This suggests that these two proteins might serve as positive markers for bovine satellite cells in FACS method. CD31 and CD45, two antibodies against endothelial cells and hematopoietic cells were utilized as negative markers for sorting satellite cells¹⁶⁻¹⁸. After isolating the mononuclear cell population obtained from bovine skeletal muscles, Hoechst was used to distinguish cells and tissue debris (Figure 1b, left). Then the CD31⁻CD45⁻CD56⁺CD29⁺ cells were isolated as bovine satellite cells (Figure 1b middle, right). *PAX7*, *PAX3* and *MYF5* mRNA were highly expressed in sorted satellite cells compared with unsorted cells or satellite cell depleted populations (Figure 1c, Supplementary Figure S1a, b). Immunofluorescent staining for PAX7 further confirmed the satellite cell identity (Figure 1d). About 92% sorted satellite cells were co-stained with DAPI and PAX7 protein (Figure 1d and 1e). We also checked other surface markers and transcriptional factors of sorted satellite cells (Supplementary Figure S1c-g), such as MYOD²⁸ (98.48 $\pm$ 1.81%), DESMIN²⁹ (98.57 $\pm$ 2.13%), M-Cadherin^{28,30} (97.64 $\pm$ 0.89%), MYF5¹⁷ (97.01 $\pm$ 2.73%), ITGA7¹⁷ (98.27 $\pm$ 3.03%). These results indicate that bovine satellite cells can be isolated from muscles tissue and purified by FACS sorting using CD31 and CD45 negative selection followed by CD56 and CD29 positive selection.

Figure 1. Isolation bovine satellite cells by specific cell surface markers

(a) Representative Immunofluorescent staining of DAPI, PAX7, CD56 (a, top), CD29 (a, bottom) on an adult bovine muscle fiber. Scale bars: 25 μ m. (b) FACS analysis of mononuclear cells from biceps femoris obtained from adult cow. Cells were gated by forward scatter and side scatter (not shown) prior to gating for Hoechst, CD45/31, CD56/29. Red gates (ovals) indicate sub-populations containing bovine satellite cells. (c) qRT-PCR analysis of PAX7 mRNA levels in total cells (not sorted), sorted satellite cells and satellite cells depleted populations after a period of 4 days in culture. (n=3). (d) Representative Immunofluorescent staining of PAX7 in sorted bovine satellite cells cultured for 4 days. Scale bars: 25 μ m. (e) Quantification of PAX7 immunofluorescent staining of sorted CD56⁺CD29⁺ cells and unsorted cells cultured for 4 days (n=3). Data are represented as mean $\pm$ SEM. Significance was analyzed by Student's t-test for 2 groups, One-way ANOVA with Bonferroni's Multiple Comparison Test for more than 2 groups. Asterisks: *** indicates $P < 0.001$.

p38 inhibition maintains PAX7 expression in bovine satellite cells

To investigate if phosphorylated (activated) p38 (p-p38) is up-regulated during culturing of bovine satellite cells, we stained the p-p38 together with PAX7 in muscle fiber and cultured bovine satellite cells (Figure 2a). In muscle fibers, only 4.6% of total PAX7⁺ cells (n=44) is p-p38⁺ as opposed to cultured satellite cells, where 95.7% of PAX7⁺ cells (n=140) are p-p38⁺. Phosphorylated-p38 was absent from quiescent satellite cells in bovine muscle fibers (Figure 2a, top); it was activated during cell culture and expansion (Figure 2a, bottom). Inhibition of p38 (p38i) MAPK signaling by SB203580 concentration-dependently increased the expression of *PAX7* mRNA (Figure 2b), whereas the solvent had no effect. Up to a concentration of 10 μ M, the p38 inhibitor had no effect on cell proliferation measured by EdU incorporation (Figure 2c). Effective p38 inhibition by SB203580 was shown by significantly reduced phosphorylation of the p38 substrate HSP27 (Ser82)^{18,24} (Figure 2d, 2e and Supplementary Figure S2a, 2b). However, in confluent cell culture, the p-p38/p38 ratio was only slightly decreased by p38i treatment (Figure 2d, 2e). These results show that p38 MAPK signaling is activated in bovine satellite cells during culture and that short-term inhibition of p38 MAPK signaling maintains the *PAX7* expression suggesting that stemness is preserved.

p38 inhibition reversibly interferes with myoblast differentiation

In order to establish p38i effects on the ability of bovine myoblasts to differentiate into myocytes, we cultured bovine satellite cells with different concentrations of the p38 inhibitor. Similar to human¹⁸, bovine satellite cells automatically fused to form myotubes upon reaching confluency (Figure 3a, left top, 3d). However, myotube formation was inhibited in p38i treatment groups (Figure 3a, 3d). At 10 μ M and 20 μ M, SB203580 strongly inhibited myotube formation (Figure 3a, 3d). *MYOGENIN* (*MYOG*) and *Myosin Heavy Chain* (*MyHC*), both markers of myocyte differentiation, were also inhibited by p38i in a concentration-dependent manner (Figure 3b, 3c). The proliferative potential of bovine satellite cells as undifferentiated cells was maintained in p38i treatment group (Supplementary Figure S3a, 3b). Immunofluorescent staining for Pax7 showed that the percentage of Pax7 positive cells was also higher in p38i treatment group (Supplementary Figure S3a, 3b). Cell lysates of 6 days cultured satellite cells accumulate more p-HSP27, MyHC proteins in the control group than in p38i treatment group (Figure 3e-g). However, the p-p38/p38 ratio showed only a slightly increase. To test whether the inhibition was reversible, we cultured cells with 10 μ M p38i for 4 days until confluency and then induced differentiation with differentiation medium in the absence of p38i. Prior p38i treated satellite cells fused into myotubes to the same degree as in the control group (Supplementary Figure S3e) suggesting that the effect on differentiation is reversible. Taken together, p38i reversibly inhibits bovine satellite cells differentiation in a concentration-dependent way.

Figure 2. *p38 inhibition maintains PAX7 expression in bovine satellite cells*

(a) Representative immunofluorescent staining of DAPI, PAX7 and p-p38 in quiescent bovine satellite cells (freshly isolated fiber, top) and activated satellite cells in culture (bottom). Scale bars: 25 μm. (b) qRT-PCR: concentration-dependent inhibition of PAX7 expression in 2 days cultured satellite cells (passage 2) by p38i inhibitor SB203580. (n=3). (c) Relative percentage of 1.5 hr window EdU incorporation at given concentrations of p38i for satellite cells at passage 2 after 2 days culturing. (n=4). (d) Representative images of immunoblotting against p-HSP27, p-p38, p38, and GAPDH from cell lysates of cells cultured 4 days to 90% confluent in the presence or absence of 10 μM p38i. Full-length blots are presented in Supplementary Figure S5. (e) Relative levels of p-HSP27 normalized to GAPDH are indicated from (d). (n=3). (f) Relative levels of p-p38 normalized to p38 from (d). (n=3). Data are represented as mean ± SEM. Significance was analyzed by Student's t-test for 2 groups, One-way ANOVA with Bonferroni's Multiple Comparison Test for more than 2 groups. Asterisks: * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$.

Figure 3. *p38 inhibition interferes with myoblast differentiation*

(a) Representative phase contrast images of passage 2 bovine satellite cells cultured for 6 days at given concentrations of p38i (SB203580). Scale bars: 200 μ m. (b) MYOG expression after 5 days of culture at given concentrations of p38i. (n=3) (c) MyHC expression after 5 and 8 days of culture at given concentrations of p38i. (n=3). (d) Representative immunofluorescent staining of DAPI and MyHC after 6 days of culture in the presence or absence of p38i. Scale bars: 50 μ m. (e) Representative images of immunoblotting against p-HSP27, p-p38, p38, MYOG and MyHC and GAPDH from cell lysates of cells cultured 6 days in the presence or absence of 10 μ M p38i. Full-length blots are presented in Supplementary Figure S5. (f) Relative levels of p-HSP27 and MyHC normalized to GAPDH are indicated from (d). (n=3). (g) Relative levels of p-p38 normalized to p38 from (d). (n=3). Data are represented as mean $\pm$ SEM. Significance was analyzed by Student's t-test for 2 groups, One-way ANOVA with Bonferroni's Multiple Comparison Test for more than 2 groups. Asterisks: * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$. N/A: not available.

Long-term p38 inhibition delays loss in stemness of bovine satellite cells

As p38i can short-term maintain bovine satellite cells stemness in an undifferentiated state, we next checked the long-term effects of p38i on cultured bovine satellite cells. Firstly, we checked the protein expression of p-HSP27, p-p38, p38 in different passage cells. During long-term culturing in vitro, bovine satellite cells showed an up-regulated p-HSP27 expression (Supplementary Figure S4a). Cells could be propagated logarithmically while long-term exposure to p38 inhibition supported bovine satellite cell proliferation (Figure 4a, 2-way ANOVA with time and treatment as independent variables: $P < 0.0001$ for time effect, $P < 0.0001$ for p38i treatment effect, $P < 0.0001$ for interaction effect). CD56 and CD29 expression were also analyzed by FACS. Inhibition of p38 maintained CD56 expression compared with the control group (Figure 4b). The expression of *PAX7* mRNA decreased in both groups during passages (Figure 4c). However, p38i resulted in higher *PAX7* expression levels compared to controls (Figure 4c, 2-way ANOVA with passage and treatment as independent variables: $P < 0.0001$ for passage effect, $P < 0.0001$ for p38i treatment effect, $P < 0.0001$ for interaction effect). We further ascertained *PAX7* positivity of cells in each passage (Supplementary Figure S4b). Consistently, the percentage of *PAX7*⁺ cells also decreased during passages whereas p38 inhibition continued to show a larger population of *PAX7*-positive cells (Figure 4d, 2-way ANOVA with passage and treatment as independent variables: $P < 0.0001$ for passage effect, $P < 0.0001$ for p38i treatment effect, $P = 0.0469$ for interaction effect). To analyze the differentiation ability of myoblasts upon passaging, we induced differentiation when cells were 90% confluence at each passage. After 10 passages, cultured myoblasts lost their capacity to differentiate into myocytes, whereas myoblasts exposed to p38 inhibition maintained this capacity (Figure 5a, b, 2-way ANOVA with passage and treatment as independent variables: $P < 0.0001$ for passage effect, $P < 0.0001$ for p38i treatment effect, $P < 0.0001$ for interaction effect). Taking together, p38 inhibition during long-term culture maintains stemness and the ability to differentiate of bovine satellite cells.

Figure 4. Long-term p38 inhibition maintains bovine satellite cells proliferation and PAX7 expression

(a) Growth curves of bovine satellite cells in absence and presence of p38i. (n=3). (b) FACS analysis of CD56 (left) and CD29 (right) expression from different passages (P2-P10) of bovine satellite cells with DMSO (top 9 lines) and p38i treatment (bottom 9 lines). (c) PAX7 mRNA levels at different passages (n=3). (d) Quantification of PAX7 immunofluorescent staining of different passages. (n=3). Data are represented as mean $\pm$ SEM. Significance was analyzed by 2-way ANOVA and Bonferroni post-tests were used to compare treatment in different passages. Asterisks: * indicates $P < 0.05$, ** indicated $P < 0.01$. *** indicates $P < 0.001$.

Figure 5. Long-term p38 inhibition delays loss in differentiation of bovine satellite cells

(a) Representative immunofluorescent images (Myosin Heavy Chain staining) of myotubes differentiated from P1, P5 and P10 satellite cells. Green indicates MYHC; blue indicates DAPI (nuclei). Scale bars, 75 μ m. (g) Percentage of nuclei in myotubes differentiated from satellite cells depending of passage (P1, P3, P5, P7, P9, P10). (n=3). Data are represented as mean $\pm$ SEM. Significance was analyzed by 2-way ANOVA and Bonferroni post-tests were used to compare treatment in different passages. Asterisks: ** indicates $P < 0.01$.

Discussion

Maintenance of stemness in a stem cell population is of considerable scientific and societal importance. Recently tissue engineering endeavors, mostly for medical, but even more so for food applications, rely on high expansion numbers of undifferentiated cells that at the same time retain their differentiation capacity^{18,19,31-33}. A prerequisite for maintaining stemness is to start with a highly purified stem cell population, since most other cells in culture grow faster than the stem cells, so that the latter will eventually be outnumbered^{16,18,34,35}. Even in a highly purified stem cell population, the more differentiated off-spring from asymmetric division, such as myoblasts from satellite cells, will overgrow the stem cells^{16,18,36}.

Here, we firstly improved the purity of bovine satellite cells by FACS and established that a combination of negative selection for CD31 and CD45 with a positive selection for CD29 and CD56 results in a high purity satellite cells population as judged by their degree of PAX7 positivity. In this highly purified population, we observed that inhibition of p38 phosphorylation kept the stem cell profile of bovine satellite cells for more passages, while they retained the ability to differentiate into myocytes.

p38 α / β MAPKs is required for satellite cells activation and can function as a molecular switch for satellite cell activation even before *MyoD* up-regulation³⁷. Because of serum and growth factor stimulation, p38 α / β signaling will continually be activated. Activated p38 α / β targets the SWI-SNF chromatin-remodeling complex to *Myog* loci³⁸, which makes the satellite cells generate more differentiated Pax7-MYOG⁺ daughter cell^{24,39,40}. The MYOG expression leads to down-regulation of genes involved in cell cycle progression and promotes differentiation and fusion⁴¹. This can be seen as a first step towards myoblast differentiation. At the same time, p38 α represses PAX7 through interaction between the transcription repressors YY1 and Polycomb Repressive Complex (PRC2)^{42,43}, further leading to loss of satellite cell stemness. Modest inhibition of p38 α will maintain PAX7 expression (Figure. 2b)^{18,24,40} and therefore the stemness of satellite cells⁴⁴⁻⁴⁶. Total inhibition of p38 α / β by SB203580 at 20 μ M also inhibited satellite cells proliferation (Figure 2c) and this is consistent with previous studies^{18,37}. At lower concentrations however, and in the presence of FGF-2, p38 inhibition slightly increased proliferation of satellite cells in long-term culture (Figure 4a), most likely due to maintenance of stemness as Pax7 expression is preserved and MYOG, as a marker of differentiation, is decreased. MYOG downregulation by SB203580 has been observed in other studies and in different cell culture models as well^{24,38,40}. Together the data show that p38 inhibition keeps satellite cells in an undifferentiated and proliferative state.

Although PAX7 decrease is delayed by p38 inhibition, long-term cultivation of bovine satellite cells still leads to loss of PAX7 expression and also loss of differentiation abilities. (Figure 4,5). Other interventions may be required to further improve the replicative capacity of satellite cells. In mouse muscle stem cells for instance, the stiffness of the

substrate elasticity helped maintaining the stemness of muscle stem cells^{24,47-49}. The suitable stiffness for bovine muscle stem cells still needs to be investigated. Another optimal way for keeping the stemness of bovine satellite cells is to culture the bovine satellite cells in hypoxia conditions⁵⁰⁻⁵². Hypoxia (1% oxygen) stimulates the proliferation and differentiation of bovine myoblasts⁵¹. Similar results have been shown in mouse satellite cells: Hypoxia (1% oxygen) conditions favor quiescence of primary mouse myoblasts by upregulating *PAX7*⁵².

We noticed progressive loss of stemness (*PAX7* positivity) during passaging, but also loss of differentiation capacity. Loss of differentiation capacity into myocytes suggests de-differentiation of satellite cells that progresses further than just losing stemness. It is currently unknown what is responsible for this de-differentiation. One option is that the cells transdifferentiate into another lineage. For instance, satellite cells can generate brown fat cells during long-term cultivation⁵³⁻⁵⁵. The *Prdm16* gene controls a brown fat and skeletal muscle switch^{53,54}. Whether this switch or other factors are responsible for the general decrease in differentiation capacity of satellite cells and myoblasts in culture, requires further studies.

We conclude that FACS purification of bovine satellite cells using CD29 and CD56 as positive markers increase the stem cell population and that in long-term culture p38 inhibition preserves stemness, proliferation and differentiation of bovine muscle precursors. This might bring large scale bovine muscle cell culture for cultured beef applications closer to reality.

Materials and Methods

Bovine muscle tissues

The bovine satellite cells in this study were derived from fresh (within 30 min of euthanasia) muscle samples obtained at a local slaughterhouse.

Bovine satellite cells isolation

Bovine satellite cells were isolated from 1-2 year old male cattle as previously described and adapted to bovine tissues^{15,16,18}. Briefly, freshly harvest bovine muscle was immediately transferred to the lab on ice and washed with 75% ethanol for 1min, followed by PBS for 2 times. Then, the tissues were mechanically dissected and dissociated with collagenase II (Worthington, CLS-2, 0.2%) in DMEM (Invitrogen, Cat# 41966-29) supplemented with 1% penicillin-streptomycin (P.S. Lonza, Cat# 17-745E) at 37°C for 1.5 h. The mixture was mixed by vertexing or triturated with pipette once per 10 min. After digestion, 20% FBS in DMEM was added and mixed well with pipette. The muscle fragments were centrifuged at 80g for 3 min and the supernatant was collected as mononuclear cell suspension. The precipitated debris was again triturated

with a 20-gauge needle in PBS and centrifugated at 80g for 3min. The supernatant was collected again and mixed with former mononuclear cell suspensions. After centrifugation at 1,000g for 5 min, the cells were washed twice with PBS followed by DMEM with 20% FBS. After that, the cells were filtered through a 100 μ m cell strainer followed by a 40 μ m cell strainer. The cells were then centrifuged at 1,000g for 5 min at 4°C and incubated with the erythrocyte lysis buffer (ACK) buffer for 5 min on ice. Then the cells were washed twice with PBS and cell pellet was reconstituted with FACS buffer (1 % BSA in PBS) or frozen in FBS supplement with 10% dimethyl sulphoxide (DMSO, sigma).

The frozen cells were first recovered in a 37°C water bath and washed with PBS twice. The cells were resuspended in FACS buffer and stained with APC anti-human CD29 Antibody (1:10, BioLegend, Cat# 303008), PE-CyTM7 anti-human CD56 (1:10, BD, Cat# 335826), FITC anti-sheep CD31 (1:10, BIO-RAD, Cat# MCA1097F), FITC anti-sheep CD45 (1:10, BIO-RAD, Cat# MCA2220F) for 30-45min on ice. After antibody incubation, the cells were washed twice with cold PBS and reconstituted in F-10 with 20% FBS. The viable CD31⁺CD45⁺CD56⁺CD29⁺ cells were isolated by cell sorting. The negative cells were also isolated to extract RNA. Cell sorting was performed with a BD FACS Aria cell sorter using 405, 488nm and 640nm lasers. Unstained cells were routinely used to define FACS gating parameters.

Satellite cell culture and differentiation

Dishes (Corning) were coated with 0.05% bovine collagen type I (Sigma, Cat# C4243). FACS isolated bovine satellite cells and unsorted cells were cultured on collagen-coated dishes in F10 medium (Gibco, Cat# 31550-023) containing 20% fetal bovine serum (FBS, Gibco, Cat# 10500-06), 5 ng/mL bFGF (R&D, Cat# 233-FB-025) and 1% P.S. Where indicated, medium was supplemented with p38i (SB203580, Selleck, Cat# S1076) and DMSO (Sigma, Cat# D8418). For serial expansion, cells were passaged to maintain a density of <60% confluence and counted at each passage. Bovine satellite cells differentiation was induced at 90% confluency with DMEM (Invitrogen, Cat# 41966-29) with 2% FBS. The expanded and differentiated cells were fixed with 4% PFA for immunofluorescent staining. The bright field images were acquired by AMG-EVOS microscope.

Immunofluorescent analysis of cultured cells

Cultured satellite cells were fixed with ice-cold 4% PFA (in PBS) for 20 min, rinsed with PBS, and permeabilized in 0.5% Triton X-100 (in PBS) for 15 min. Permeabilized cells were blocked and incubated with primary antibodies in 1% BSA (in PBS) overnight at 4°C. Primary antibodies recognizing mouse Pax7 (1:10, Developmental Studies Hybridoma Bank, Cat# Pax7), mouse myosin heavy chain (1:100, Sigma, Cat# M4276), mouse MyoD (1:200, ABclonal, CAT# A0671), mouse Desmin (1:100, sigma, Cat# D1033), mouse M-cadherin (1:200, BD, Cat# 611100), Rabbit Myf5 (1:50, Santa Cruz,

Cat# SC-302), Rabbit ITGA7 (1:100, LifeSpan BioSciences, Cat# LS-C313325), Rabbit p-p38 (1:100, Cell Signaling, Cat#9211). After washing with PBS, cells were incubated with Alexa 488 labeled anti-mouse (1:600, Invitrogen, Cat #A-11001) antibodies for 1h at room temperature and mounted with VECTASHIELD mounting medium with DAPI (Vector Laboratories Cat# H-1500).

EdU detection was carried-out using a Click-It EdU detection kit (Life Technologies, Cat# C10337) according to the manufacturer's instructions.

For the FACS analysis of cultured satellite cells, about 10^5 cells were collected at each passage. The cells were fixed with 4% PFA for 15 min and washed two times for 5min with PBS. The cells were then incubated with Alexa Fluor 488 anti-human CD29 (1:10, BioLegend, Cat#303016), PE-conjugated anti-human CD56 (1:10, BioLegend, Cat# 304606) in 1% BSA in PBS for 30-45 min. After antibody incubation, the cells were washed with PBS for two times and reconstituted in PBS. FACS analysis was performed with the Cytomics FC500 Flow Cytometer (Beckman Coulter) cell analyzer. Unstained cells were routinely used to define FACS gating parameters.

Western blots analysis of cultured cells

Western blots were either performed from total cell lysates obtained by lysing cells directly with RIPA buffer complemented with PMSF, protease inhibitor cocktail and sodium orthovanadate (Santa Cruz, CAT# sc-24948). Proteins concentration was determined using BCA protein assay kit (Thermo). SDS-PAGE electrophoresis was carried out in 7.5% pre-cast polyacrylamide gels (Bio-Rad, CAT# 5671023). After transfer onto nitrocellulose, membranes were blocked for 30 min with Odyssey Blocking Buffer in PBS (Part Number: 927-40000, LI-COR Biosciences) and probed overnight with primary antibody: p-HSP27(Ser 82) (1:500, Cell Signaling, CAT# 9709), p-p38 (1:1000, Cell Signaling, CAT# 9211), p38 (1:1000, Cell Signaling, CAT# 8690), MyHC (1:500, Millipore, CAT# 05-716), GAPDH (1:500, Millipore, CAT# MAB374). After that, IRDye700-conjugated or IRDye800-conjugated secondary antibodies were used and visualized with the Odyssey infrared detector (LI-COR Biosciences, Westburg, Leusden, the Netherlands). For Protein quantification a, we used the background correction option in the software of the supplier (Image Studio™ Software for the Odyssey CLx-LI-COR Biosciences) and scanned the corresponding band of the protein of interest.

Bovine muscle fiber isolation and analysis

The bovine muscle fiber was isolated as previously described and adapted to bovine tissues¹⁵. After procurement, part of the specimen was immediately fixed in 4% paraformaldehyde (PFA) at room temperature for 20 min and washed with PBS. The sample was then embedded in 30% glycerol in PBS overnight at 4°C, then 50% glycerol in PBS overnight at 4°C, then 80% glycerol in PBS overnight at 4°C and finally 100% glycerol. The fibers were stored in 100% glycerol at 4°C until dissection.

Single fibers were dissected using fine forceps under a dissecting microscope. Single fibers were washed in PBS for 15 min at room temperature, then permeabilized with 0.5% Triton X-100 (Sigma-Aldrich, Cat#X-100) for 10 min, and washed twice with PBS for 8min. Single fibers were then blocked with 3% goat serum in PBS for 1h at room temperature and incubated overnight at 4°C with the following primary antibodies: mouse monoclonal anti-Pax7 (1:10 Developmental Studies Hybridoma Bank, Cat#Pax7), p-p38 (1:100, Cell Signaling, Cat#9211), The next day, after PBS wash at room temperature for 15 min, fibers were incubated with Alexa 488 labeled anti-mouse (1:600, Invitrogen, Cat#A-11001), Alexa 594 labeled anti-rabbit (1:600, Invitrogen, Cat#:A-21207) antibodies for 1h at room temperature. Then mounted with VECTASHIELD mounting medium with DAPI (Vector Laboratories Cat# H-1500). All images were acquired by Leica SP6 confocal microscope and processed with Adobe Photoshop CS5 to adjust brightness and contrast for publication.

For fibers co-stained with Pax7 and CD56/CD29, Pax7 staining was firstly performed. After incubation with the secondary antibody (Alexa 488 labeled anti-mouse for Pax7 and CD29 co-staining, Alexa 647 labeled anti-mouse (1:600, Invitrogen, Cat#:A-31571) for Pax7 and CD56 co-staining) above, sections were washed twice with PBS for 8 min and incubated with the APC anti-human CD29 (1:10, BioLegend, Cat# 303016) or PE anti-human CD56 (1:10, BioLegend, Cat# 304606) antibody for 2h at room temperature. After washing with PBS for 3 times, fibers were mounted with VECTASHIELD mounting medium with DAPI (Vector Laboratories, Cat# H-1500). All images were acquired by Leica SP6 confocal microscope and processed with Adobe Photoshop CS5 to adjust brightness and contrast for publication.

Gene expression analysis

RNA was extracted from cells using the RNeasy Micro Kit (QIAGEN, Cat#74004) including RNase-Free DNase Set (QIAGEN, Cat# 79254) according to the manufacturer's instruction. 500 ng of total RNA from each sample was reverse transcribed to cDNA using iScript cDNA Synthesis Kit (Bio-Rad, Cat#1708891) according to the manufacturer's instruction. Relative gene expression was performed in triplicate using a SYBR Green PCR master mix on an CFX 96 Real Time PCR system (Bio-Rad).

The primers used in these assays were the followings:

PAX7-F, 5'- CTCCCTCTGAAGCGTAAGCA -3', *PAX7*-R, 5'- GGGTAGTGGGTCCTCTCGAA -3';
PAX3-F, 5'- CAAAGCTTACAGAGGCCCGA -3', *PAX3*-R, 5'- GGTCTCTGACAGCTGGTACG -3'
MYF5-F, 5'- TCTATCTCTCTGCTGTCCAGGC -3', *MYF5*-R, 5'- AACTCGTCCCCGAACCTCAC -3';
MYOG-F, 5'- GCGCAGACTCAAGAAGGTGA -3', *MYOG*-R, 5'- TGCAGGCGCTCTATGTACTG -3';
MyHC-F, 5'-AGAGCAGCAAGTGGATGACCTTGA-3',
MyHC-R,5'- TGGACTCTTGGGCCAACTTGAGAT -3';
GAPDH-F, 5'- CACCCTCAAGATTGTCAGC -3', *GAPDH*-R, 5'- TAAGTCCCTCCACGATGC -3'.

Pax7⁺ cells population, differentiation efficiency, EdU and pHSP27 percentage measurement

Pax7⁺ cell populations were expressed as the number of nuclei co-stained with DAPI and Pax7 divided by the total number of nuclei in the same field. More than 300 nuclei from 5 or more randomly chosen fields were analyzed.

The percentage of myotube nuclei per condition was expressed as the nuclei inside myotube (at least 3 nuclei) divided by total nuclei in the same field. More than 1000 nuclei from 4 randomly chosen fields were analyzed.

For EdU detection, cells cultured in BD Falcon 96 wells HTS Imaging microplates were imaged using a High-Content Analyzer (BD Pathway 855) with a 4× objective. In total 16 images were taken for each condition and individual cell segmentation and analysis was performed using the BD AttoVision software (BD Biosciences, version 1.6). Hoechst positive cells were segmented and counted, then the same was done for EdU positive cells. The numerical data were further analyzed with Kaluza software (version 1.3; Beckman Coulter), where the EdU positive percentage was determined for more than 6000 nuclei from 16 randomly chosen fields.

For pHSP27(Ser82) detection, cells cultured in 96-well plate were imaged using a High-Content Imager (BD Pathway 855) with a 20× objective. Together 16 images were taken for each condition. Using AttoVision-software, the entire cell was segmented by fluorescence intensity and the pHSP27 (Ser82) positive speckles were counted in each cell. The average speckles were defined between different groups. More than 700 nuclei from 16 randomly chosen fields were analysed.

Statistics

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software). For comparisons of two treatment groups, a Student's t-test was used. For more than two groups, One-way ANOVA with Bonferroni's Multiple Comparison Test was used. For long-term culture experiments, 2-way ANOVA was used to distinguish the p38i treatment and passages effects. Results were means ± S.E.M. unless otherwise stated. $P < 0.05$ was considered significant.

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Supplementary information

Figure S1. Characterization of sorted bovine satellite cells. Related to Figure 1

(a) qRT-PCR analysis of *PAX3*, *MYF5* mRNA levels in total cells (not sorted), sorted satellite cells and satellite cells depleted populations after a period of 4 days in culture ($n=3$). (b) Immunofluorescent staining of Pax7 and MYOD in sorted bovine satellite cells cultured for 4 days. Scale bars: 25 μ m. (c) Immunofluorescent staining of DESMIN, M-Cadherin, MYF5, ITGA7 in sorted bovine satellite cells cultured for 4 days. Scale bars: 75 μ m. Data are represented as mean $\pm$ SEM. Significance was analyzed by One-way ANOVA with Bonferroni's Multiple Comparison Test. Asterisks: *** indicates $P < 0.001$.

Figure S2. *p38i treatment repressed phosphorylation of the p38 substrate HSP27 (Ser82). Related to Figure 2*

(a) Representative IF analysis of phospho-HSP27 (Ser82) in passage 2 bovine cells cultured for 2 days in the presence or absence of p38i. Scale bar: 100 μ m (b) Quantification of IF for phospho-HSP27 (Ser82) per cell from (a). (n=3). Data are represented as mean $\pm$ SEM. Significance was analyzed by Student's t-test. Asterisks: *** indicates $P < 0.001$.

Figure S3. *p38i* treatment maintains the proliferation and Pax7 population of satellite cells. Related to Figure 3

(a) Representative DAPI and EdU immunofluorescent staining of 1 h window EdU incorporation after 6 days of culture in the absence (top panels) or presence (bottom panels) of p38i. Scale bars: 50µm. (b) Relative percentage of EdU from (a). (c) Representative immunofluorescent staining of DAPI and Pax7 after 6 days of culture in the absence (top panels) or presence (bottom panels) of p38i. Scale bars: 75µm. (d) Quantification of Pax7 immunofluorescent staining from (c). (e) Representative phase contrast images of differentiated bovine satellite cells. Passage 2 satellite cells are originally cultured in F-10 medium with DMSO or 10µM p38i for 4 days before differentiation. Scale bars:400µm. Data are represented as mean ± SEM. Significance was analyzed by Student's t-test. Asterisks: ** indicates $P < 0.01$.

Figure S4. *p38-MAPK signal is up-regulated during long-term culturing and p38i treatment maintains PAX7 population. Related to Figure 4*

(a) Representative images of immunoblotting against p-HSP27, p-p38, p38 and GAPDH of myoblast cells from P2, P4, P8, P10 cultured in F-10 medium. Full-length blots are presented in Supplementary Figure S5.
(b) Representative Immunofluorescent staining of PAX7 in different passage myoblast cells cultured in the presence or absence of p38i. Scale bars: 75µm.

Figure S5. Full-length western blots. Related to Figure 2, Figure3, Figure S4

(a) Representative full-length immunoblotting blots against p-HSP27, p-p38, p38 and GAPDH from cell lysates of cells cultured 4 days to 90% confluent in the presence or absence of 10 μ M p38i. (b) Representative full-length immunoblotting blots against p-HSP27, p-p38, p38, MyHC and GAPDH from cell lysates of cells cultured 6 days in the presence or absence of 10 μ M p38i. (c) Representative full-length immunoblotting blots against p-HSP27, p-p38, p38 and GAPDH of myoblast cells from P2, P4, P8, P10 cultured in F-10 medium.

Chapter 4

Regulation of STAT3 signal in differentiation and long-term culturing of bovine muscle stem cells

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Abstract

In muscle biology, the activator of transcription 3 (STAT3) pathway is not only important in regulating skeletal muscle mass, repair, aging and diseases in vivo, but also can regulate the in vitro proliferation and differentiation of myoblasts. However, some groups reported conflicting data on the function of STAT3 in the stemness of satellite cells. We show that STAT3 signal was activated in cultured bovine myoblast cells. The JAK2-STAT3 signal was required for bovine myoblast differentiation. Inhibition of JAK2-STAT3 signal impaired the differentiation process. However, JAK2-STAT3 inhibition did not maintain satellite stemness or proliferation or long-term passaging of myoblasts. Western blots results indicated that the JAK2-STAT3 signal was not the reason for the long-term culture defects in bovine myoblasts.

Introduction

Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway is known to have a critical role in cell growth, proliferation and differentiation in many cell types¹⁻³. In mammals, the JAK family kinases include four members (JAK1, JAK2, JAK3 and TYK2 i.e. Tyrosine kinase 2), whereas the STAT family has seven (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6)^{4,5}. The JAK-STAT family is activated by cytokines or growth factors, e.g. leukemia inhibitory factor (LIF) and basic fibroblast growth factor (bFGF or FGF-2)^{2,6,7}. Activated JAKs lead to phosphorylation of their selective STATs, which dissociate from the receptor, dimerize and translocate into the nucleus to regulate expression of target genes^{2,6}.

The JAK-STAT pathway is not only important in regulating skeletal muscle mass, repair, aging and diseases *in vivo*⁵, but can also regulate the differentiation of satellite cells or myoblast cells *in vitro*⁸. Following acute muscle damage, IL-6-induced activation of STAT3 signaling is essential for satellite cell activation and muscle regeneration^{9,10}. Two recent studies reported that JAK2-STAT3 signaling in muscle stem cells induce myogenic differentiation to the detriment of their expansion¹¹⁻¹³. In accordance, knockdown of STAT3 in satellite cells decreased the expression of both MYOD and Myogenin (MYOG)^{13,14}, thus demonstrating the pivotal role of STAT3 in satellite cell myogenic progression. In myoblasts, JAK1-STAT1-STAT3 plays a notable role in proliferation, whereas JAK2-STAT3 and JAK3 mainly mediate differentiation^{3,8,15}. In summary, the JAK2-STAT3 signaling has complex effects in muscle regeneration and satellite cell function.

STAT3 signalling is also involved in aging of muscle. Aged muscle shows an up-regulated IL-6/STAT3 signal^{13,16} and pharmacological inhibition of STAT3 activity promoted satellite cell expansion and enhanced tissue repair from aged muscle¹³. Long-term culture of cells may serve as an *in vitro* model of aged cells¹⁷. In this study we investigated the function of STAT3 signalling in bovine myoblast cell proliferation, differentiation, and long-term culture. We found that JAK2-STAT3 signal is essential for myoblast cells culture and differentiation. However, the JAK2-STAT3 signal is not the reason for the long-term culture defects in bovine myoblast cells.

Results

p-STAT3 is activated in bovine myoblasts during culturing

Under physiological conditions, quiescent satellite cells do not express p-STAT3. However, activated satellite cells start to express p-STAT3 during muscle injury¹³. Since the STAT3 signal pathway is up-regulated in mouse satellite cells activation^{5,18,19}, we checked the expression of p-STAT3 in cultured bovine satellite cells. Immunofluorescent staining showed that the p-STAT3 was expressed in cultured bovine satellite cells (Figure 1).

Figure 1. *p-STAT3 is activated in bovine myoblasts during culturing*

(a) Representative Immunofluorescent staining of DAPI, MYOD, p-STAT3 on bovine muscle stem cells cultured for 4 days. Scale bars: 25 μm .

JAK2-STAT3 is important for myoblast proliferation but does not maintain the stemness gene *PAX7*

In mouse satellite cells, pharmacological inhibition of JAK2 or STAT3 maintains the Pax7⁺ satellite cells per fiber in single muscle fiber culture¹⁹ and supports satellite cell proliferation in vitro¹³. To investigate pharmacological inhibition of JAK2 or STAT3 in bovine myoblast cells culturing in vitro, we cultured bovine myoblast cells with STAT3i (Stattic) and JAK2i (AG490). EdU incorporation results showed that up to 4 μM , STAT3i and JAK2i had no effect on bovine myoblast cells proliferation (Figure 2a, 2b). Higher concentrations of STAT3i (4 μM) and JAK2i (≥ 4 μM) inhibited myoblast cells proliferation (Figure 2a, 2b). The *PAX7* expression in passage 2 was not affected by the different concentrations of STAT3i and JAK2i (Figure 2c, 2d). It has been reported that MYOD is regulated by STAT3^{13,14}. We also checked the *MYOD* expression with the STAT3i and JAK2i treatment. High concentration of STAT3i (4 μM) showed *MYOD* repression whereas JAK2i treatment didn't affect *MYOD* expression in mRNA level (Figure 2e, 2f).

Figure 2. JAK2-STAT3 is important for myoblast proliferation but does not maintain the stemness gene PAX7

(a) Relative percentage of 1 hr window EdU incorporation at given concentrations of STAT3i for myoblast cells at passage 2 after overnight culture. (n=4) (b) Quantification of the percentage of EdU⁺ cells at different concentrations of JAK2i for myoblast cells at passage 2 after overnight culture. (n=4). (c, d) PAX7 relative expression at different concentrations of STAT3i (c) or JAK2i (d) by qRT-PCR. (n=3-6). (e, f) MYOD relative expression at different concentrations of STAT3i (e) or JAK2i (f) by qRT-PCR. (n=6). Data are represented as mean $\pm$ SEM. Significance was analyzed by One-way ANOVA with Bonferroni's Multiple Comparison Test. Asterisks: * indicates $P < 0.05$, ** indicates $P < 0.01$.

JAK2-STAT3 signal is essential for bovine myoblast cell differentiation

In mouse and human myoblasts, JAK2-STAT3 signal is up-regulated during myoblast fusion^{3,14,20}. We investigated if the JAK2-STAT3 signal is also increased in bovine myoblast differentiation. We induced bovine myoblast differentiation by lowering the serum to 2%. Bovine myoblasts showed nice myotubes formation after 3 days of differentiation (Figure 3a, left). The STAT3 downstream proteins SOCS3, p-JAK2, p-STAT3 were up-regulated during differentiation (Figure 3b). The addition of STAT3i and JAK2i decreased the myotube size (Figure 3a, middle and right) and associated MYHC expression (Figure 3c, 3d). We also cultured bovine myoblast cells for 8 days in growth medium. Under these conditions, bovine myoblast cells automatically fuse to form nascent myotubes upon reaching 100% confluency (Figure 4a). However, JAK2i and STAT3i treatment inhibited the myotube formation ability compared with control groups. The *Myogenin* (*MYOG*) and *MyHC*, both markers of myocyte differentiation, were also inhibited by JAK2i and STAT3i (Figure 4b, 4c). Altogether, JAK2-STAT3 signal is up-regulated during bovine myoblast differentiation. Addition of JAK2i and STAT3i inhibits the differentiation process, indicating that JAK2 and STAT3 signalling is required during differentiation.

Figure 3. *JAK2-STAT3 signal is essential for bovine myoblast cell differentiation*

(a) Representative phase contrast images of passage 2 bovine myoblast cells differentiated with control (left), STAT3i (middle), JAK2i (right). Cells were cultured to 95% confluency and then switched to differentiation medium. Scale bars: 400 μ m. (b) Representative images of immunoblotting against MYHC, SOCS3, p-STAT3, STAT3, p-JAK2, JAK2 and GAPDH from cell lysates of myoblast, 95% confluency and differentiation stage. (c) Representative images of immunoblotting against MYHC and GAPDH from cell lysates of passage 2 bovine myoblast cells differentiated with control, JAK2i, STAT3i. (d) Relative levels of MyHC normalized to GAPDH from (c). (n=1). Data are represented as mean.

Figure 4. *JAK2 and STAT3 inhibition interferes with myoblast differentiation*

(a) Representative phase contrast images of passage 2 bovine myoblast cells cultured for 8 days in growth medium with control, JAK2i, STAT3i. Scale bars: 400 μm. (b) *MYOG* expression after 7 days of culture in growth medium with control, JAK2i and STAT3i. (n=1). (c) *MyHC* expression after 7 days of culture in growth medium with control, JAK2i and STAT3i. (n=1). Data are represented as mean±SD.

In long-term culture, STAT3i and JAK2i have no effect on proliferation, stemness gene expression and differentiation of long-term culture of bovine myoblasts

Eventhough JAK2i and STAT3i doesn't affect the stemness gene *PAX7* in short term treatment, they inhibit the differentiation of bovine myoblast cells. We checked long-term culture of bovine myoblast cells with JAK2i and STAT3i. Both of the inhibitors didn't affect the proliferation of bovine myoblast cells in long-term culture (Figure 5a). The *PAX7* and *MYOD* expression were also not affected by STAT3i treatment in P6, P8 or JAK2i in P10 (Figure 5b, c). Passage 9 myoblasts in different treatment groups showed similar differentiation abilities (Figure 5d). Altogether, JAK2i or STAT3i doesn't affect proliferation, stemness gene expression or differentiation in long-term passage.

Figure 5. *STAT3i* and *JAK2i* have no effect on proliferation, stemness gene expression and differentiation of long-term culture of bovine myoblasts

Cells were serially passaged to maintain a density of <60% confluency and were counted at each passage. (a) Growth curve of bovine myoblast cells cultured with STAT3i, JAK2i or control. (n=3 for STAT3i, n=2 for JAK2i). (b) *PAX7* and *MYOD* mRNA levels at passage 6 and 8 during long-term passage with or without STAT3i. (n=1). (c) *PAX7* and *MYOD* mRNA levels at passage 10 during long-term passage with or without JAK2i. (n=1). (d) Representative phase contrast images of passage 9 myoblast cells from control (left), STAT3i (middle), JAK2i (right) were induced differentiation with the same differentiation medium. Data are represented as mean $\pm$ SEM. For the growth curve, significance was analyzed by 2-way ANOVA and Bonferroni post-tests were used to compare treatment in different passages.

JAK2-STAT3 signal is stable during long-term passage

Since the expression of p-JAK2 and p-STAT3 is very weak by Western Blot in myoblast, we analysed the total STAT3, JAK2 and downstream SOCS3 in different passages. Those proteins did not show obvious changes (Figure 6a, b). Taking together, long-term passage doesn't affect the JAK2-STAT3 signal pathway.

Figure 6. JAK2-STAT3 signal is stable during long-term passage

(a) Representative images of immunoblotting against SOCS3, STAT3, JAK2 and GAPDH from cell lysates of different passage myoblast cells during serial expansion. (b) Relative levels of SOCS3, JAK2, STAT3 proteins normalized to GAPDH from (a). Data are represented as mean $\pm$ SEM (n=3). Significance was analyzed by One-way ANOVA with Bonferroni's Multiple Comparison Test.

Discussion

In our results, we observed STAT3 activation during short term cultured of bovine myoblast cells. High concentration (more than 4μM) of JAK2i or STAT3i treatment inhibited myoblast cells proliferation. JAK2i and STAT3i did not affect stemness gene *PAX7* expression of bovine myoblast cells but inhibited the differentiation process. Pharmacological inhibition of JAK2 and STAT3 also did not affect proliferation or differentiation abilities of the bovine myoblast cells in long-term culture (Figure 6), Further protein analysis through western blotting indicates that JAK2-STAT3 signaling does not seem to determine the long-term culture defects in bovine myoblast cells.

STAT3 in proliferation

STAT3 signaling is important in myoblasts proliferation in vitro. Activated STAT3 (p-STAT3) is not expressed or in a low level in satellite cells under physiological conditions^{13,21}. After culturing in vitro, the p-STAT3 is expressed in satellite cells¹³ (Figure 1). However, the role of STAT3 in satellite cell proliferation is controversial. On one hand, LIF-induced STAT3 and STAT1 activation results in myoblast proliferation in C2C12 that is inhibited by knockdown of STAT3²². The STAT3 conditional KO satellite cells are defective in cell proliferation²³. On the other hand, others have shown that pharmacological or shRNA inhibition of STAT3 supported mouse and human satellite cells proliferation, mainly by inhibition of MYOD and MYOG expression¹³. In our hands, both JAK2i and STAT3i inhibited myoblast proliferation at high dose, suggesting that STAT3 and JAK2 activation during proliferation indeed have a small stimulatory effect in bovine myoblast cells (Figure 2a, 2b).

STAT3 in differentiation

Unlike the proliferation and in vivo data, JAK2-STAT3 signaling in differentiation is clear. During myoblast differentiation, both activated JAK2 (p-JAK) and STAT3 (p-STAT3) are up-regulated²⁴. JAK2 is critical for the MYOD and MEF2 expression and transactivation function²⁵. STAT3 phosphorylation increases during myoblast differentiation and activates SOCS-3 transcription²⁶. As a feedback, SOCS3 inhibits the IGF1-induced STAT3 activation^{27,28}. The myogenic differentiation was inhibited by knockdown of JAK2 or STAT3^{24,25}. Likewise, pharmacological JAK2i and STAT3i treatment inhibited muscle differentiation^{13,29}. In our results, bovine myoblast cells differentiation was inhibited by JAK2i and STAT3i both in growth medium (20% serum) and differentiation medium (2% serum) (Figure 4 and 5). However, optimized JAK2i and STAT3i didn't affect the differentiation ability during long-term passaging (Figure 5). STAT3 is required for muscle differentiation, but inhibition does not preclude future differentiation.

In vivo, the role of STAT3 is more complex

It is evident that JAK2-STAT3 signal is important in muscle stem cells in vivo. Damage by overloading muscle causes increased p-STAT3 positive satellite cells²¹. Likewise, loss of muscle mass in disease conditions is also due to activated STAT3 (p-STAT3), which means that p-STAT3 initiates muscle wasting^{5,30,31}. In what seems to be a contrast, however, the IL-6/STAT3 axis is also critical in satellite cell proliferation-mediated hypertrophic muscle growth³².

A similar dichotomy is observed in muscle injury models. Injury induced IL-6-activated STAT3 signaling results in defective regeneration. Transient STAT3 inhibition by STAT3 inhibitors promotes satellite cell expansion and enhances tissue repair in both young, aged and dystrophic muscle^{12,13}. It should be noted that the chemical inhibitors used in the studies above may have off-target effects^{5,23}. Conditional ablation of STAT3 signal in satellite cells results in their increased expansion during regeneration¹³. Similarly, STAT3 conditional KO in satellite cells by another group show opposite results in that the satellite cells decrease after muscle injury²³. In fact, the former group only finds the increased expansion of satellite cells in 2-month old but not in 3-month old MuSC-specific STAT3 knockout mice¹³. Irrespective of the age effect, both groups showed that MuSC-specific STAT3 knockout mice showed impaired regeneration. Those results confirm a complex effect of STAT3 signal in satellite cells in vivo, which needs more research to clarify.

STAT3 signal in aging

In transcriptome profiles higher expression of JAK2-STAT3 signaling in aging muscle stem cell has been established^{12,33}. Inhibition of JAK2-STAT3 signaling rescues the proliferation and engraftment defect of satellite cells from the muscle of older adult mice¹². The reason for up-regulation of JAK2-STAT3 signal in aging satellite cells could be increased inflammation as indicated by a higher IL-6 signal^{16,30}. Likewise, in elder muscle, increased IL-6 activated STAT3, SOCS3 protein expression^{30,34-36}. Subculture of cells may be viewed as an aging model¹⁷ and long-term cultured satellite cells show some similarities with aging satellite cells in vivo^{37,38}. In subculture of TIG3 human fibroblasts, aged cells (later passages) showed higher p-STAT3 signaling than younger cells³⁹. However, in two other reports, it was found that the p-STAT3 expression was high in young passages as well^{40,41}. In our long-term cultured myoblast cells, we didn't observe an up-regulation of STAT3 during passaging.

Therefore, our results show that JAK2-STAT3 signal is important in bovine myoblast cells proliferation and differentiation. However, the JAK2-STAT3 signal is not the reason for the long-term culture defects in bovine myoblast cells.

Materials and Methods

Bovine muscle tissues

The bovine satellite cells in this study were derived from fresh muscle samples obtained at a local slaughterhouse. The transport time is about 30 min.

Bovine satellite cells isolation

Bovine satellite cells were isolated from 4-6 year old male cattle as previously described and adapted to bovine tissues⁴²⁻⁴⁴. The detailed method for isolation mononuclear cells from muscle tissue was described in Chapter 2.

The frozen cells were first recovered in growth medium (F10 + 20% fetal calf serum + 5ng/ml bFGF) for 2 days. The floating debris were washed away and the adherent cells were collected by trypsin dissociation. These cells were resuspended in FACS buffer and stained with APC anti-human CD29 Antibody (1:10, BioLegend, Cat# 303008), PE-CyTM7 anti-human CD56 (1:10, BD, Cat# 335826), FITC anti-sheep CD31 (1:10, BIO-RAD, Cat# MCA1097F), FITC anti-sheep CD45 (1:10, BIO-RAD, Cat# MCA2220F) for 30-45min on ice. After antibody incubation, the cells were washed twice with cold PBS and reconstituted in growth medium. The viable CD31⁺CD45⁺CD56⁺CD29⁺ cells were isolated by cell sorting. Cell sorting was performed with a BD FACSAriaII cell sorter using 405, 488nm and 640nm lasers. Unstained cells were routinely used to define FACS gating parameters.

Satellite cell culture and differentiation

Dishes (Corning) were coated with 0.05% bovine collagen type I (Sigma, Cat# C4243). FACS isolated bovine satellite cells and unsorted cells were cultured on collagen-coated dishes in F10 medium (Gibco, Cat# 31550-023) containing 20% fetal bovine serum (FBS, Gibco, Cat# 10500-06), 5 ng/mL bFGF (R&D, Cat# 233-FB-025) and 1% P.S. Where indicated, medium was supplemented with STATTIC (Sigma, Cat# S7947), AG490 (InvivoGene, Cat# tlrl-ag4) and DMSO (Sigma, Cat# D8418). For serial expansion, cells were passaged to maintain a density of <60% confluence and counted at each passage. Bovine myoblast cells differentiation was induced at 95% confluency with DMEM (Invitrogen, Cat# 41966-29) with 2% FBS. The expanded and differentiated cells were fixed with 4% PFA for immunofluorescent staining. The bright field images were acquired by AMG-EVOS microscope.

Immunofluorescent analysis of cultured cells

Cultured satellite cells were fixed with ice-cold 4% PFA (in PBS) for 20 min, rinsed with PBS, and permeabilized in 0.5% Triton X-100 (in PBS) for 15 min. Permeabilized cells were blocked and incubated with primary antibodies in 1% BSA (in PBS) overnight at 4°C. Primary antibodies recognizing rabbit MYOD (1:200, ABclonal, CAT# A0671),

mouse STAT3 (1:50, cell signaling technology, CAT# 9139). After washing with PBS, cells were incubated with Alexa 488 labeled anti-rabbit (1:600, Invitrogen, Cat # A-11034), 594 labeled anti-mouse (1:600, invitrogen, Cat # A-11032) antibodies for 1h at room temperature and mounted with VECTASHIELD mounting medium with DAPI (Vector Laboratories Cat# H-1500).

EdU detection was carried-out using a Click-It EdU detection kit (Life Technologies, Cat# C10337) according to the manufacturer's instructions.

Western blots analysis of cultured cells

Western blots were either performed from total cell lysates obtained by lysing cells directly with RIPA buffer complemented with PMSF, protease inhibitor cocktail and sodium orthovanadate (Santa Cruz, CAT# sc-24948). Proteins concentration was determined using BCA protein assay kit (Thermo). SDS-PAGE electrophoresis was carried out in 7.5% pre-cast polyacrylamide gels (Bio-Rad, CAT# 5671023). After transfer onto nitrocellulose, membranes were blocked for 30 min with Odyssey Blocking Buffer in PBS (Part Number: 927-40000, LI-COR Biosciences) and probed overnight with primary antibody: p-STAT3 (1:250, Cell Signaling, CAT# 9138S), STAT3 (1:500, Cell Signaling, CAT# 9139S), p-JAK2 (1:250, Cell Signaling, CAT# 3776S), JAK2 (1:500, Cell Signaling, CAT# 3230S), SOCS3 (1:500, Cell Signaling, CAT# 2932S), MyHC (1:500, Millipore, CAT# 05-716), GAPDH (1:500, Millipore, CAT# MAB374). After that, IRDye700-conjugated or IRDye800-conjugated secondary antibodies were used and visualized with the Odyssey infrared detector (LI-COR Biosciences, Westburg, Leusden, the Netherlands). For Protein quantification a, we used the background correction option in the software of the supplier (Image Studio™ Software for the Odyssey CLx-LI-COR Biosciences) and scanned the corresponding band of the protein of interest.

Gene expression analysis

RNA was extracted from cells using the RNeasy Micro Kit (QIAGEN, Cat#74004) including RNase-Free DNase Set (QIAGEN, Cat# 79254) according to the manufacturer's instruction. 500 ng of total RNA from each sample was reverse transcribed to cDNA using iScript cDNA Synthesis Kit (Bio-Rad, Cat#1708891) according to the manufacturer's instruction. Relative gene expression was performed in triplicate using a SYBR Green PCR master mix on an CFX 96 Real Time PCR system (Bio-Rad).

The primers used in these assays were the followings:

PAX7-F, 5'- CTCCCTCTGAAGCGTAAGCA -3', PAX7-R, 5'- GGGTAGTGGGTCCTCTCGAA -3';
 MYOD-F, 5'- CGACGGCATGATGGACTACA-3', MYOD-R, 5'- GTAAGTGCGGTCGTAGCAGT -3'
 MYOG-F, 5'- GCGCAGACTCAAGAAGGTGA -3', MYOG-R, 5'- TGCAGGCGCTCTATGTACTG -3';

MyHC2-F, 5'-AGAGCAGCAAGTGGATGACCTTGA-3',

MyHC-R,5'- TGGACTCTGGGCCAACTTGAGAT -3';

GAPDH-F, 5'- CACCCTCAAGATTGTCAGC -3', GAPDH-R, 5'- TAAGTCCCTCCACGATGC -3'.
EdU percentage measurement

For EdU detection, cells cultured in BD Falcon 96 wells HTS Imaging microplates were imaged using a High-Content Analyzer (BD Pathway 855) with a 10× objective. In total 16 images were taken for each condition and individual cell segmentation and analysis was performed using the BD Attovision software (BD Biosciences, version 1.6). Hoechst positive cells were segmented and counted, then the same was done for EdU positive cells. The numerical data were further analyzed with Kaluza software (version 1.3; Beckman Coulter), where the EdU positive percentage was determined for more than 1000 nuclei from 16 randomly chosen fields.

Statistics

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software). For comparisons of two treatment groups, a Student's t-test was used. For more than two groups, One-way ANOVA with Bonferroni's Multiple Comparison Test was used. Results were means ± S.E.M. unless otherwise stated. $P < 0.05$ was considered significant.

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Chapter 5

Loss of stemness in satellite cells during subculturing is distinct from loss of stemness by differentiation

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Abstract

Satellite cells are quiescent in uninjured muscle tissue and can quickly enter the cell cycle following muscle injury or culturing in vitro. On one hand, satellite cells easily start myogenic differentiation with irreversibly withdrawal from the cell cycle when cultured in vitro. On the other hand, satellite cells also lose their capability to multiply when they are serially subcultured in the absence of differentiation. Both of these two processes lead to loss of stemness of satellite cells. Whether differentiation is the main reason for the subculture defects is discussed here. Here we compare several proteins in crucial signaling pathways during differentiation and long-term culture. Differentiation and long-term culture showed a similar up-regulation of the p38-MAPK signal. In contrast, the JAK2-STAT3 signal pathway was up-regulated during differentiation but remained stable during long-term culture. Both ERK and Akt signals were down-regulated when the cells reached confluency to subsequently increase during differentiation. During long-term subculturing ERK signaling kept decreasing whereas Akt signal continued to increase. Our results show that differentiation and long-term passaging are two different biological processes at the level of major signaling pathways. Preventing cell differentiation in early stage myocytes or myoblasts is not sufficient to maintain the stemness of satellite cells during long-term passaging.

Introduction

Satellite cells are a population of small mononucleated muscle stem cells^{1,2}. They are about 2-10% of the total amount of myonuclei^{3,4}. In physiological conditions, satellite cells are quiescent and are characterized by the expression of the paired box protein 7 (PAX7)¹. They are activated upon injury and start asymmetric division to generate the myogenic progenitors needed for muscle regeneration^{1,5}. The other daughter cells from asymmetric division do not commit to differentiation, and can undergo self-renew to replenish the muscle stem cell pool⁵.

Satellite cells have been successfully isolated and cultured in vitro⁶⁻⁸. Even though the activated satellite cells in vivo show different gene expression profiles compared with satellite cells activated after cell culture^{9,10}, they still share the myogenic process. The satellite cell is activated in part by the induced expression of MYOD and MYF5^{5,11-15}. During activation, satellite cells (or myoblasts) start to express myogenic determination protein (MYOD) and myogenic factor 5 (MYF5)^{11,16-18}. Further differentiation is regulated by the downregulation of PAX7 and de novo expression of MYOGENIN (MYOG)^{5,19,20}. The terminally differentiated myotube expresses myosin heavy chain (MyHC) and muscle specific creatin kinase (MCK)²⁰.

Several signals are up-regulated during activation, such as p38 MAPK^{10,21,22} and JAK-STAT signals²³⁻²⁵, which mediate the transition of satellite cells to more differentiated PAX7⁻MYOG⁺ daughter cells, aka myoblasts²⁶⁻²⁸. The MYOG expression leads to down-regulation of genes involved in cell cycle progression and promotes differentiation and fusion²⁹. The subsequent automatic differentiation impairs the expansion of satellite cells or myoblasts in vitro³⁰⁻³².

Subculturing of stem cells decreases the proliferation and differentiation abilities, especially in mesenchymal stem cells³³⁻³⁶. The defects after serial cultivation have also been observed in myoblast cells³⁷⁻³⁹. Whether the automatic differentiation is the main reason for subculture defects is still unclear in satellite cells.

Here we present data to show that subculturing and differentiation are two different biological processes. They share up-regulation of the p38-MAPK signal. However, the JAK2-STAT3, ERK and Akt signals were differently expressed in long-term culture compared with the differentiation process. Preventing the cell to differentiate in early stage myocytes/myoblasts is not sufficient to maintain the stemness of satellite cells during long-term passaging.

Results

Differentiation and long-term culture of bovine myoblasts

Bovine myoblast cells were cultured to confluency and induced to differentiate by 2% fetal bovine serum. These cells formed multinuclear myotubes (Figure 1a). Myosin heavy chain protein increased in differentiated myotubes, which confirmed the differentiation process (Figure 1b, 1c). The long-term passage cells showed larger cell size during passaging (Figure 1d). The MYOD protein was up-regulated during passaging and maintained stable in later passages (Figure 1e, 1f).

p38 MAPK signaling

It has been reported that p38-MAPK signaling is up-regulated during differentiation and aging^{27,28,40-43}. We compared the p38-MAPK signal in these two conditions at the protein level. Differentiation resulted in an up-regulated phosphorylation of the specific p38 downstream target protein HSP27 (Figure 2a, 2b)^{44,45}. Phosphorylation of p38 however did not change (Figure 2a, 2b). The long-term passage also showed up-regulation of HSP27 phosphorylation (Figure 2c, 2d). The phosphorylation of p38 showed a slight upward trend with increasing passage number. Total p38 level did not change (Figure 2c, 2d). Both differentiation and longterm passage showed up-regulation of the p38-MAPK signal (Figure 2e-g).

Figure 1. Differentiation and long-term culture of bovine myoblasts

(a) Representative phase contrast images of P3 bovine myoblast cells at proliferation (50% confluency), pre-differentiation (95% confluency) and differentiation stage. Scale bars: 200 μ m. (b) Representative images of immunoblotting against MyHC and GAPDH from cell lysates of myoblast, 95% confluency and differentiation stage. (c) Relative levels of MyHC normalized to GAPDH are indicated from (b). (n=3). Data are represented as mean $\pm$ SEM. Significance was analyzed by One-way ANOVA with Bonferroni's Multiple Comparison Test. Asterisks: * indicates $P < 0.05$. (d) Representative phase contrast images of P3, P6, P9 bovine myoblast cells. Scale bars: 200 μ m. (e) Representative images of immunoblotting against MYOD and GAPDH from cell lysates of myoblast at passage 2, 4, 8, 10, 12. (f) Relative levels of MYOD normalized to GAPDH are indicated from (b). (n=1).

Figure 2. *p38 MAPK signaling in differentiation and subculturing processes*

(a) Representative images of immunoblotting against p-HSP27, p-p38, p38 and GAPDH from cell lysates of myoblast, 95% confluency and differentiation stage. (b) Relative levels of p-HSP27, p-p38, p38 normalized to GAPDH are indicated from (a). (n=3). (c) Representative images of immunoblotting against p-HSP27, p-p38, p38 and GAPDH from cell lysates of myoblast at passage 3, 6, 9. (d) Relative levels of p-HSP27, p-p38, p38 normalized to GAPDH are indicated from (c). (n=3). (e, f, g) The combination results from (b) and (d) for p-HSP27 (e), p-p38 (f), p38 (g). Data are represented as mean $\pm$ SEM. Significance was analyzed by One-way ANOVA with Bonferroni's Multiple Comparison Test. Asterisks: * indicates $P < 0.05$.

JAK2-STAT3 signaling

The JAK2-STAT3 signal is up-regulated during myoblast fusion^{23,46,47}. Aged satellite cells also show up-regulated JAK2-STAT3 signal⁴⁸. In accordance, the levels of p-JAK2 and p-STAT3 up-regulated during differentiation (Figure 3a, 3b, chapter 4). SOCS3 is downstream of STAT3 signaling and can create a negative feedback to prevent the induction of gp130 signaling⁴⁹⁻⁵¹. SOCS3 gradually increased during differentiation⁵⁰. Our results also showed a higher expression of SOCS3 during differentiation (Figure 3a, 3b). However, SOCS3 expression did not change during long-term passaging (Figure 3c, 3d). Since the expression of p-JAK2 and p-STAT3 is very weak, we checked total JAK2 and STAT3 proteins and they were not affected by passage number either (Figure 3c, 3d). Thus, differentiation and long-term passaging are associated with different expression patterns of SOCS3, JAK2 and STAT3 signaling (Figure 3e-3f).

Akt signaling

Akt signaling can regulate the balance between protein synthesis and degradation in muscle mass and is also involved in cell survival^{52,53}. When primary bovine myoblasts reach confluence, the p-Akt showed a lower expression level compared with non-confluent myoblasts (Figure 4a, 4b). However, Akt phosphorylation was up-regulated again when cell fusion started as the initial step towards muscle differentiation (Figure 4a, 4b). Similar results were also presented in C2C12 cell lines that moved from myoblast to final differentiation^{24,46,54}. Total Akt showed a slight up-regulation during differentiation (Figure 4a, 4b). However, the long-term passage cells showed a different p-Akt expression profile. p-Akt was increased during long-term passaging while total Akt didn't change (Figure 4c, 4d). Thus, the expression of Akt is different between differentiation and long-term passage (Figure 4e, 4f).

Figure 3. *JAK2-STAT3 signaling in differentiation and subculturing processes*

(a) Representative images of immunoblotting against SOCS3, p-JAK2, JAK2, p-STAT3, STAT3 and GAPDH from cell lysates of myoblast, 95% confluency and differentiation stage. (b) Relative levels of SOCS3, p-JAK2, JAK2, p-STAT3, STAT3 normalized to GAPDH are indicated from (a). (n=3). (c) Representative images of immunoblotting against SOCS3, JAK2, STAT3 and GAPDH from cell lysates of myoblast at passage 3, 6, 9. (d) Relative levels of SOCS3, JAK2, STAT3 normalized to GAPDH are indicated from (c). (n=3). (e, f, g) The combination results from (b) and (d) for SOCS3 (e), JAK2 (f), STAT3 (g). Data are represented as mean $\pm$ SEM. Significance was analyzed by One-way ANOVA with Bonferroni's Multiple Comparison Test (differences were non-significant).

Figure 4. Akt signaling in differentiation and subculturing processes

(a) Representative images of immunoblotting against p-Akt, Akt and GAPDH from cell lysates of myoblast, 95% confluency and differentiation stage. (b) Relative levels of p-Akt, Akt normalized to GAPDH are indicated from (a). (n=3). (c) Representative images of immunoblotting against p-Akt, Akt and GAPDH from cell lysates of myoblast at passage 3, 6, 9. (d) Relative levels of p-Akt, Akt normalized to GAPDH are indicated from (c). (n=3). (e, f) The combination results from (b) and (d) for p-Akt (e), Akt (f). Data are represented as mean $\pm$ SEM. Significance was analyzed by One-way ANOVA with Bonferroni's Multiple Comparison Test (differences were non-significant).

ERK signaling

ERK activation has been reported to mediate myoblast proliferation⁵⁵. In accordance with a previous report⁵⁶, p-ERK was down-regulated when the primary bovine myoblasts reached confluence and then increased in late differentiation (Figure 5a, 5b). Total ERK remained unchanged (Figure 5a, 5b). During long-term passaging, p-ERK decreased with passage number whereas total ERK increased (Figure 5c, 5d). The ERK signal is different expressed between differentiation and long-term passage (Figure 5e, 5f).

Other relevant signaling pathways

Leukemia inhibitory factor (LIF) is known to stimulate myoblast proliferation and inhibit its differentiation^{50,57-59}. However, LIF did not support the proliferation of bovine myoblast cells in long-term culture (Figure 6a-c).

The canonical Wnt/ β -catenin signal is important in myoblast differentiation^{30,60}. Inhibition of canonical Wnt/ β -catenin induces defects in myoblast fusion^{30,60}. Inhibition of Setd7 by PFI-2, which cause failure of β -catenin to accumulate in the nucleus upon Wnt stimulation allows the in vitro expansion of myogenic stem cells with improved therapeutic potential³⁰. However, our results showed that PFI-2 failed to support long-term bovine myoblast culture (Figure 6d). Non-canonical WNT signaling by WNT7A promotes symmetric cell divisions in vivo and cultured fibers^{61,62} and should therefore maintain stemness of satellite cells. WNT7A has also been reported to induce long-lasting effects on cell migration of myoblasts leading to in vitro hypertrophy⁶²⁻⁶⁴. In our conditions, in primary bovine myoblasts, WNT7A did not support long-term passaging (Figure 6e).

These divergence of results with LIF and WNT in long-term passaging versus reported positive effects on differentiation, further underscore that failure to be extensively subcultured is likely not a result of differentiation of the myoblasts.

Figure 5. ERK signaling in differentiation and subculturing processes

(a) Representative images of immunoblotting against p-ERK, ERK and GAPDH from cell lysates of myoblast, 95% confluency and differentiation stage. (b) Relative levels of p-ERK, ERK normalized to GAPDH are indicated from (a). (n=3). (c) Representative images of immunoblotting against p-ERK, ERK and GAPDH from cell lysates of myoblast at passage 3, 6, 9. (d) Relative levels of p-ERK, ERK normalized to GAPDH are indicated from (c). (n=3). (e, f) The combination results from (b) and (d) for p-ERK (e), ERK (f). Data are represented as mean $\pm$ SEM. Significance was analyzed by One-way ANOVA with Bonferroni's Multiple Comparison Test.

Figure 6. Preventing cell differentiation is not sufficient to maintain the stemness during long-term passaging

(a) Relative percentage of 1 hr window EdU incorporation at different concentrations of LIF for myoblast cells at passage 2 after overnight culture without bFGF. (n=4). (b) Relative percentage of 1 hr window EdU incorporation at different concentrations of LIF for myoblast cells at passage 2 after overnight culture with bFGF. (c) Growth curve of bovine myoblast cells cultured with LIF (5ng/ml) or control. Cells were serially passaged to maintain a density of <60% confluency and were counted at each passage. (d) Growth curve of bovine myoblast cells cultured with PFI-2 (4μM) or control. Cells were serially passaged to maintain a density of <60% confluency and were counted at each passage. (e) Growth curve of bovine myoblast cells cultured with WNT7A (50ng/ml) or control. Cells were serially passaged to maintain a density of <60% confluency and were counted at each passage.

Discussion

Differentiation of myoblasts is stimulated by high cell density and reduction of nutrition, i.e. serum factors^{65,66}. In the long-term passage conditions, the cells were cultured at less than 60% confluence and with sufficient nutrition. Even in those conditions there is a gradual loss of proliferation and differentiation ability during long-term culture similar to the changes occurring during aging^{27,67}. Long-term culture of cells may serve as an *in vitro* model of aged cells⁶⁸. Several intracellular processes and signaling pathways have been described in relation to the dysregulated regenerative capacity in aging satellite cells *in vivo* such as mitochondrial dysfunction^{69,70}, bFGF and FGF receptor 1 function²⁷, protein homeostasis (proteostasis)⁷¹, p38^{27,28}, STAT3^{32,72}, p16⁷³, canonical WNT⁷⁴ and Notch signaling⁷⁵, as well as epigenetic status^{76,77}. Only a few studies focus on myoblast aging during long-term culture³⁷⁻³⁹.

Here, we showed several signal pathway changes in long-term culturing (as a model for aging) and in differentiation by Western blot. Expression patterns in differentiation and aging were similar in p38-MAPK signaling but different in JAK2-STAT3, ERK and Akt signaling.

5

1. p38-MAPK signaling

1.1 p38-MAPK in differentiation

p38 signal is very important during differentiation, which was discussed before (Chapter 3).

1.2 p38-MAPK in aging

In old animals, satellite cells show a senescent phenotype and an autonomous loss in regeneration, self-renewal and engraftment ability^{27,28,71,73,78}. This could be mediated by alterations in p38 α / β MAPK signaling in satellite cells from aged mice^{27,28} as p38 α / β MAPK inhibition rescues proliferation and augments stem cell gene expression in satellite cells from aged mice, thus rejuvenating their potential for regeneration^{27,28}. The same result is obtained by pharmacologic inhibition of p38 α / β in young satellite cells in mice and human²⁸. Aging results in elevated p38 α / β signaling, possibly from increased cellular stress and inflammatory responses^{27,68,77}.

Long-term culture is generally considered an *in vitro* model of cellular aging^{68,79,80}. This has been, at least partly, related to oxidative stress (ROS production) induced senescence in MSC cells^{68,81-83}. The elevated ROS levels have been suggested to increase stress signaling, such as p38-MAPK, that leads to loss of stem cell function^{77,84}. The impaired phosphorylation activity of p38 MAP kinase (p38 MAPK) in long-term cultured hMSCs led to a compromised immunomodulatory ability through the regulation of COX-

²⁸⁵. Elevated ROS levels and a senescent phenotype were also found in aged muscle stem cells in vivo^{71,73}. Whether aging and differentiation are similar in our long-term cultured bovine myoblast with respect to p38-MAPK signaling still needs to be shown. In accordance with earlier aging studies of satellite cells, in long-term passage, our bovine myoblast cells showed up-regulation of the p38 MAPK signal. At a functional level, p38 MAPK appears to be elevated as the p38 substrate p-HSP27 was increased during passaging (Figure 2), so it is highly likely that in bovine myoblasts p38 signaling also leads to senescence and inhibition of stem cell function. Indeed, we have shown in an earlier study that inhibition of p38 partially protects against loss of satellite stem cell function during long-term passaging (Chapter 3)⁸⁶.

2. STAT3 signaling

During myoblast differentiation, both activated JAK2 (p-JAK) and STAT3 (p-STAT3) are up-regulated⁵⁰. STAT3 phosphorylation activates SOCS-3 transcription during myoblast differentiation⁵¹. The STAT3 signal is also up-regulated during aging in vivo^{69,72}. However, conflict data are shown by subculturing process⁸⁷⁻⁸⁹. The STAT3 signal in differentiation and aging was discussed before (Chapter 4)

3. Akt signaling

3.1 Akt signaling in differentiation

PI3K/Akt is up-regulated during differentiation^{90,91} and induces muscle differentiation by increasing the transcriptional activity of MyoD and MEF2C^{90,92}. PI3K/Akt also regulates MYOG expression through p70S6K during differentiation⁹⁰. Conversely, inhibition of PI3K/Akt signaling down-regulates the MYOG expression thus inhibiting differentiation^{54,90,91}. In addition, increased Akt signal dominantly inhibits the effects of myostatin, which also leads to the differentiation^{93,94}. In contrast with the general opposing patterns in proliferation and differentiation, p-Akt is expressed at higher levels during early passage proliferation than in the first 12h (early differentiation stage) of differentiation⁴⁶. Our results showed a similar expression pattern. p-Akt expression was high in the myoblast proliferation stage, down-regulated when cells were confluent, but up-regulated again during differentiation (Figure 4).

3.2 Akt signaling in aging

Akt signaling pathway has physiological responses fundamental to healthy aging and longevity^{95,96}. PI3K/Akt is persistently activated in aging muscle and neuronal tissue in Alzheimer's disease (AD)^{95,97}. Reducing, but not obliterating, the IGF-1/PI3-K/Akt signal can extend a healthy lifespan in organisms from yeast to mammals (reviewed in^{95,96}). This effect is an evolutionarily conserved influence on somatic aging and longevity⁹⁶. During long-term passaging, increased Akt signal is also responsible for the replicative

senescence in cultured human endothelial cells and vascular smooth muscle cells^{98,99}. Akt inactivates the FoxO3a-SOD2 axis and consequently allows ROS production, which elicits senescence through irreparable DNA damage^{100,101}. The activated Akt signal is also increased in our long-term passage, but if this is a cause or effect of senescence in bovine myoblast still needs to be established by intervention studies.

4. ERK signaling

4.1 ERK signaling in proliferation and differentiation

The ERK expression is high during the myoblast (proliferation) stage (Figure 5), especially when bFGF is added to the medium. Blocking ERK1/2 activity through inactivation of the Raf-MKK1/2-ERK1/2 pathway in myoblasts prevents myoblast proliferation⁵⁵. The bFGF stimulated ERK1/2 signaling is required during the G1 phase of the cell cycle and thus important for proliferation but is not required for either repression or promotion of differentiation⁵⁵. However, others found that ERK showed a biphasic activation profile from myoblast to myotube^{102,103} with peaks in ERK1/2 activity in undifferentiated myoblasts as well as in postmitotic myotubes. When the differentiation starts, the activation of ERK1/2 inhibits the differentiation stage¹⁰². There is a critical time window (16–20h) for bFGF to activate ERK and to repress myogenic gene expression and differentiation¹⁰⁴. For efficient terminal differentiation of skeletal myoblast cells, a final activation of ERK2 is required¹⁰³. In accordance, our own observations also show biphasic regulation of ERK activity during myogenic differentiation (Figure 5).

4.2 ERK signaling in aging

The ERK signal in replicative senescence by long-term passaging has been well studied. ERK signal is decreased during long-term passage^{105,106}. The suppression of MEK/ERK pathway in senescent cells leads to accumulation of damaged mitochondria and reactive oxygen species (ROS)¹⁰⁵. Dephosphorylation of ERK1/2 leads to p53-mediated upregulation of MKP-3, which contributes to the senescent phenotype¹⁰⁷. The MKP2-mediated inactivation of nuclear ERK2 is also a key event in replicative senescence¹⁰⁸. Restoration of nuclear ERK activity can extend replicative lifespan¹⁰⁸. ERK is indispensable for myoblast proliferation^{55,103}. In accordance, our results with primary bovine myoblasts showed down-regulation of p-ERK in late passages (Figure 5). Whether sustained ERK activation is an achievable and effective target for maintain the long-term culture needs to be confirmed with gain of function experiments.

Other mediators of differentiation

Two additional pathways that are frequently studied in the setting of muscle differentiation are LIF and WNT. Proteins from the IL-6 family such as LIF, CT-1, inhibit myogenic differentiation and repress skeletal myogenesis through interference with

Myogenic regulatory factors (MRF) activity by activation of MEK/ERK signaling^{47,109-111}. This repression is independent of JAK-STAT signaling. In contrast however, LIF enhances transplantation efficiency of satellite cells suggesting preservation of differentiation capacity¹¹². We studied LIF during the proliferation phase of myoblasts and we did not find an effect on proliferation (Figure 6). Differentiation as a function of LIF was not studied but we may expect enhanced differentiation based on other studies.

Canonical WNT activation is shown to drive differentiation of myoblasts. Induction of Canonical WNT signaling by Setd7 enhances in vitro expansion of satellite cells³⁰. Likewise, non-canonical WNT signaling by WNT7A promotes myoblast expansion in vivo and in cultured myofibers^{61,62}. WNT7A also induces long-lasting effects on cell migration that finally support the myoblast to participate in muscle repair⁶²⁻⁶⁴. In our in vitro conditions, inhibiting the canonical WNT pathway with PFI-2 (an inhibitor of Setd7) or stimulation of the non-canonical by WNT7A did not affect long-term passaging so in vitro their roles are still unclear (Figure 6).

To conclude, our results showed that subculturing and differentiation are two different biological processes. Although the profiles of p38-MAPK signaling were similar during differentiation and long-term culture, JAK2-STAT3, ERK and Akt signals showed clear differences between the kinase activities during these two processes. Inhibition of differentiation is not sufficient for maintaining the stemness during long-term passaging. Future work is needed to clarify the biological changes during long-term passaging of bovine myoblasts and to develop interventions that support prolonged cell culture.

Materials and Methods

Bovine muscle tissues

The bovine satellite cells in this study were derived from fresh muscle samples obtained at a local slaughterhouse. The transport time is about 30 min.

Bovine satellite cells isolation

Bovine satellite cells were isolated from 4-6 year old male cattle as previously described and adapted to bovine tissues^{8,31,113}. The detailed method for isolation mononuclear cells from muscle tissue was described in Chapter 2.

The frozen cells were first recovered in growth medium (F10 + 20% fetal calf serum + 5ng/ml bFGF) for 2 days. The floating debris were washed away and the adherent cells were collected by trypsin dissociation. These cells were resuspended in FACS buffer and stained with APC anti-human CD29 Antibody (1:10, BioLegend, Cat# 303008), PE-CyTM7 anti-human CD56 (1:10, BD, Cat# 335826), FITC anti-sheep CD31 (1:10, BIO-

RAD, Cat# MCA1097F), FITC anti-sheep CD45 (1:10, BIO-RAD, Cat# MCA2220F) for 30-45min on ice. After antibody incubation, the cells were washed twice with cold PBS and reconstituted in growth medium. The viable CD31⁺CD45⁺CD56⁺CD29⁺ cells were isolated by cell sorting. Cell sorting was performed with a BD FACSAriaII cell sorter using 405, 488nm and 640nm lasers. Unstained cells were routinely used to define FACS gating parameters.

Satellite cell culture and differentiation

Dishes (Corning) were coated with 0.05% bovine collagen type I (Sigma, Cat# C4243). FACS isolated bovine satellite cells and unsorted cells were cultured on collagen-coated dishes in F10 medium (Gibco, Cat# 31550-023) containing 20% fetal bovine serum (FBS, Gibco, Cat# 10500-06), 5 ng/mL bFGF (R&D, Cat# 233-FB-025) and 1% P.S. Where indicated, medium was supplemented with PFI-2 (Sigma, Cat# SML1408), LIF (R&D Systems, Cat# 7734-LF) and DMSO (Sigma, Cat# D8418). For serial expansion, cells were passaged to maintain a density of <60% confluence and counted at each passage. Bovine myoblast cells differentiation was induced at 95% confluency with DMEM (Invitrogen, Cat# 41966-29) with 2% FBS and 1% P.S. The bright field images were acquired by AMG-EVOS microscope.

Immunofluorescent analysis of cultured cells

EdU detection was carried-out using a Click-It EdU detection kit (Life Technologies, Cat# C10337) according to the manufacturer's instructions.

Western blots analysis of cultured cells

Western blots were either performed from total cell lysates obtained by lysing cells directly with RIPA buffer complemented with PMSF, protease inhibitor cocktail and sodium orthovanadate (Santa Cruz, CAT# sc-24948). Proteins concentration was determined using BCA protein assay kit (Thermo). SDS-PAGE electrophoresis was carried out in 7.5% pre-cast polyacrylamide gels (Bio-Rad, CAT# 5671023). After transfer onto nitrocellulose, membranes were blocked for 30 min with Odyssey Blocking Buffer in PBS (Part Number: 927-40000, LI-COR Biosciences) and probed overnight with primary antibody: mouse MyoD (1:200, ABclonal, CAT# A0671), Buffer in PBS (Part Number: 927-40000, LI-COR Biosciences) and probed overnight with primary antibody: p-HSP27(Ser 82) (1:500, Cell Signaling, CAT# 9709), p-p38 (1:1000, Cell Signaling, CAT# 9211), p38 (1:1000, Cell Signaling, CAT# 8690), p-STAT3 (1:250, Cell Signaling, CAT# 9138S), STAT3 (1:500, Cell Signaling, CAT# 9139S), p-JAK2 (1:250, Cell Signaling, CAT# 3776S), JAK2 (1:500, Cell Signaling, CAT# 3230S), SOCS3 (1:500, Cell Signaling, CAT# 2932S), Erk (1:250, Cell Signaling, CAT# 9107S), p-Erk (1:1000, Cell Signaling, CAT# 4370S), Akt (1:500, Cell Signaling, CAT# 4685S), p-Akt (1:1000, Cell Signaling, CAT# 4060S), MyHC (1:500, Millipore, CAT# 05-716), GAPDH (1:500, Millipore, CAT# MAB374). After that, IRDye700-conjugated or IRDye800-conjugated secondary antibodies were used and

visualized with the Odyssey infrared detector (LI-COR Biosciences, Westburg, Leusden, the Netherlands). For Protein quantification a, we used the background correction option in the software of the supplier (Image Studio™ Software for the Odyssey CLx-LI-COR Biosciences) and scanned the corresponding band of the protein of interest.

EdU percentage measurement

For EdU detection, cells cultured in BD Falcon 96 wells HTS Imaging microplates were imaged using a High-Content Analyzer (BD Pathway 855) with a 10× objective. In total 16 images were taken for each condition and individual cell segmentation and analysis was performed using the BD Attovision software (BD Biosciences, version 1.6). Hoechst positive cells were segmented and counted, then the same was done for EdU positive cells. The numerical data were further analyzed with Kaluza software (version 1.3; Beckman Coulter), where the EdU positive percentage was determined for more than 1000 nuclei from 16 randomly chosen fields.

Statistics

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software). For more than two groups, One-way ANOVA with Bonferroni's Multiple Comparison Test was used. Results were means $\pm$ S.E.M. unless otherwise stated. $P < 0.05$ was considered significant.

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Chapter 6

Proteomics of bovine muscle stem cell aging

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Abstract

Muscle stem cells (MuSCs; also known as satellite cells) are an ideal cell source to produce cultured meat. However, upon long-term culturing, bovine satellite cells eventually show proliferation and differentiation defects, referred to as loss of stemness. The defects caused by serial subculture has parallels with cell senescence and might therefore serve as an *in vitro* aging model. MuSCs have a specific aging phenotype defined by biomarkers that are also found in aged (murine) donors. We used label-free high-throughput proteomics to identify the protein changes in early and late passages to characterize the aging process and to identify potential candidates to prevent aging and with it the proliferation and differentiation defects. Further gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis showed that the proteins up-regulated in the late passages are involved in cell-cell adhesion, muscle contraction, mitochondrial electron transport NADH to ubiquinone, cell redox homeostasis, Fatty acid degradation, PI3K-Akt signaling pathway and Insulin signaling pathway. Early passages showed high expression of proteins involved in DNA replication, protein folding, translational initiation, DNA repair, fibroblast growth factor receptor signaling pathway, p53 regulation, proteasome-ubiquitin-dependent proteolysis, telomere maintenance via recombination and somatic stem cell population maintenance. These proteomic profiles correlate with the aging signal of MuSCs *in vivo* and relicative senescence signals *in vitro*. The proteomics data indicate that the dysfunction of satellite cells during long-term passage resembles aging.

Introduction

Aging is defined as the time-dependent persistent change of cellular (and also systemic) functionality and reproducibility, which affects most living organisms^{1,2}. Aging research is becoming more and more popular and has experienced an unprecedented advance over recent years¹⁻³. There are two cellular aging models². Cells isolated from an old donor are called *in vivo* aged cells, which can be directly used for aging studies. On the other hand, *in vitro* aged cells can be obtained by prolonged subculturing or by stressing the cells with noxious substances such as oxidants, AGEs or lipofuscin². Both models provide insight into the aging process.

Satellite cells are the stem cells in muscle tissue which are responsible for muscle regeneration upon injury^{4,5}. They are located in a stem cell niche between the sarcolemma and the basement membrane of adult skeletal muscle cells. Aged MuSCs are decreased in cell number, and show impaired self-renewal and loss of regenerative ability upon implantation in *in vivo* muscle injury models⁶⁻⁸. The changes in extracellular niche function and intrinsic cell properties lead to MuSC dysfunction⁷⁻⁹. Several intracellular processes and signaling pathways have been described as being dysregulated in association with aging of satellite cells *in vivo* such as mitochondrial dysfunction^{10,11}, FGF2 and FGF receptor 1 function¹², protein homeostasis (proteostasis)¹³, p38^{12,14}, STAT3^{15,16}, p16¹⁷, canonical Wnt¹⁸, Notch signaling¹⁹, and epigenetic status^{8,20}.

The limited replicative life-span of human fibroblast upon prolonged culturing was first described in 1961 and was further described as replicative senescence²¹⁻²³. It is also referred to as *in vitro* aging of cells since replicative senescence showed similarities with *in vivo* aging². Long-term cultured myoblast cells not only show proliferation arrest²⁴⁻²⁷, but are also impaired with respect to their ability to differentiate²⁸. One of the features of cells subject to extensive subculturing is gradual impairment of glucose and lipid metabolism^{26,27}. Long-term cultured myoblast cells *in vitro* also quickly and dramatically downregulate telomerase activity²⁹. However, more information on ageing satellite cells *in vitro* is still needed^{26,27}.

Quantitative proteomics, which is based on mass spectrometry, is a powerful approach used to understand global changes in protein expression in a cell, tissue or organism³⁰. It can identify the protein expression and modifications underlying the molecular mechanisms of biological processes and disease states. Several studies used two-dimensional differential in-gel electrophoresis (2D-DIGE) in myoblast aging *in vitro* as a method to separate proteins by size and charge^{27,31}. 2D-GE, however, has a number of inherent drawbacks, the major being that it is fairly insensitive to low copy proteins and thus cannot be used for the entire proteome^{32,33}. In recent years, gel-free proteomics have become more popular due to their simpler experimental designs and sample-preparation methods^{30,33}. The developed quantitative proteomics has matured and is capable of delivering accurate, reproducible and comprehensive data at high throughput³⁰.

Understanding the molecular process controlling muscle stem cell proliferative expansion and commitment to differentiated cell lineages during long-term culture is crucial to obtain large amounts of functional cells for regenerative medicine and cultured meat research. Using the proteomics method, we identified some pathways that are increased and decreased during long-term passage. The proteomics data indicate that the dysfunction of satellite cells during long-term passage resembles the effect of aging.

Results

Subculture of bovine myoblast cells induce defects in proliferation and differentiation

Satellite cells gradually lose their stemness when cultured in vitro^{24-26,34}. During long-term passaging, the cells showed increased cell size (Figure 1a). Subculturing of myoblast cells also resulted in decreased proliferation as indicated by the cell doubling rate per day (Figure 1b). We also checked the differentiation ability of an early passage (P3) and late passage (P9). After undergoing a differentiation protocol, P3 cells were 80% MyHC⁺ cells whereas only 15% of cells from P9 were MyHC positive (Figure 1c, 1d). These results indicate that long-term passaging leads to a gradual loss function of myoblast cells in proliferation as well as differentiation.

Quantitative proteomics showed good quality data

To understand the reason for loss of stemness during passaging, we collected the cells from P3, P6, P9 and applied an MS-based label-free proteomics quantitative method. After filtering with master and high FDR confidence protein, 1874 reliable quantitative proteins were obtained across all passages in at least 3 of 4 replicates. The sample abundance of the proteins were similar for the groups and replicates suggesting sufficient quality of sample preparation and protein identification (Figure 2a). To assess the variability and reproducibility between replicates and passages, we next generated a principle component analysis (PCA). The PCA plot showed that the P3, P6, P9 were nicely separated by principle component 1 (34.6%). The quantitative proteomics data from the four biological replicates correlated very well, while showing distinct differences between the passages (Figure 2b). Finally, we checked the expression of several housekeeping proteins. GAPDH, β -tubulin isotypes TUBB2A and TUBB2B were constant in different passages (Figure 2c). Taking together, these results indicate sufficient quality of proteomics data.

Figure 1. Bovine myoblast cells lose the ability to proliferate and to differentiate after long-term culture

(a) Representative phase contrast images of bovine myoblast cells cultured in F-10 medium at passage 3, 6, 9. Scale bars: 200 μ m. (b) Bovine myoblast cell division times in 24 hrs at different passages. Data are represented as mean $\pm$ S.E.M. (n=3–8). (c) Representative immunofluorescent staining images of Myosin Heavy Chain staining of myotubes differentiated from P3 and P9 myoblast cells after differentiation. Green indicates MYHC; blue indicates DAPI (nuclei). Scale bars: 200 μ m. Significance was analyzed by Student's t test or one-way ANOVA followed by Dunnett's Multiple Comparison Test. Asterisks: *** indicates $p < 0.001$.

Figure 2. Quantitative proteomics shows good quality data

(a) The abundance of the peptides within the proteins in the samples are shown. Sample abundance was generated by PD 2.2 according to the quantitative peptides. Data are represented as mean $\pm$ SD. (b) Principal component analysis (PCA) of all 1874 quantified proteins. Using principal components 1 and 2, 4 biology replicates in passage 3, 6, 9. Blue indicates 4 biological replicates of P3. Orange indicates 4 biological replicates of P6. Green indicates 4 biological replicates of P9. (c) Pooled housekeeping proteins GAPDH, β -tubulin isotypes TUBB2A and TUBB2B abundances in passage 3, 6, 9. Data are represented as mean $\pm$ SD. None of the differences were significant (one-way ANOVA)

The quantitative proteins can be divided in 3 different clusters

We generated a heatmap using the 4 replicates for different passages. The dendrogram showed results similar to the PCA plot (Figure 3a). The heatmap of pooled proteins show different profiles for each passage (Figure 3b), which is especially obvious if one clusters the proteins according to magnitude of difference between the passages (Figure 3c). Cluster 1 contained protein ratios were 1.2 or more in passage 9 compared with passage 3. Proteins that had an abundance ratio of 0.8 or less in P9 compared with P3 comprised cluster 2, whereas cluster 3 represented the proteins stably expressed in P3 and P9. The P9 to P3 abundance ratios were between 0.8-1.2 (Figure 3c). Since we were interested in the changes during longterm passage, we focussed on clusters 1 and 2.

DAVID analysis of enriched proteins in Cluster 1

In cluster 1, with proteins that were up-regulated upon passaging, 328 proteins were enriched. Gene ontology (GO) was performed to identify any potential functional biological role of those proteins using the online Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources. Gene Ontology Biological Process annotation (GO_BP) showed that cell-cell adhesion, muscle contraction, mitochondrial electron transport, NADH to ubiquinone and cell redox homeostasis were up-regulated during passaging (Figure 4a). The KEGG PATHWAY analysis was also performed and some new pathways like Phagosome, Fatty acid degradation, PI3K-Akt signaling pathway and Insulin signaling pathway were found (Figure 4b). Those proteins were also related to aging diseases such as Parkinson's disease, Alzheimer's disease and Huntington's disease according to KEGG PATHWAY. These results indicate that long-term passaging increased mitochondrial and redox stress, imbalanced cell homeostasis and aging related defects.

Figure 3. Clustering of quantitative proteins

(a) Heatmap of all quantified proteins expression profile in 4 biology replicates from passage 3, 6, 9. The dendrogram showed the variety and unity between replicates and passages. (b) Heatmap of pooled all quantified proteins expression profile from passage 3, 6, 9. (c) The quantitative proteins are divided into 3 clusters of according to protein expression between passage 3 and 9. Pooled proteins abundance ratios are 1.2 or more (left), 0.8 or less (middle), between 0.8 and 1.2 (right) from passage 9, as compared with passage 3.

Figure 4. Signaling pathways enriched in Cluster 1

DAVID Analysis demonstrated up-regulated signaling pathways in bovine myoblast cells from passage 9, as compared with passage 3 using Gene Ontology Biological Process annotation (GO_BP) enrichment and KEGG pathway enrichment. (a) GO_BP analysis of 328 enriched proteins in cluster 1. (b) KEGG pathway analysis of 328 enriched proteins in cluster 1. Signaling pathways were ranked on the basis of Benjamini-Hochberg corrected p-value. Each dot represents a signal pathway. Specific pathways related to satellite cell function are highlighted in red.

DAVID Analysis of enriched proteins in Cluster 2

In total 443 proteins were highly expressed in passage 3 in cluster 2, GO_BP analysis showed that DNA replication, protein folding, translational initiation, DNA repair, fibroblast growth factor receptor signaling pathway, regulation of signal transduction by p53 class mediator, proteasome-mediated ubiquitin-dependent protein catabolic process, telomere maintenance via recombination and somatic stem cell population maintenance were enriched in P3 cells (Figure 5a). In addition, KEGG pathway analysis also showed that mismatch repair and ubiquitin mediated proteolysis pathways were highly expressed in p3. These results indicate that higher expression of proteostasis, fibroblast growth factor receptor, p53 signal and telomere maintenance could help maintain the proliferation and differentiation abilities of myoblast cells in P3 (Figure 5b).

Long-term cultured bovine myoblasts showed alert mitochondrial proteins expression

Proteins that were commonly up-regulated during passaging showed a substantial overlap (48 genes) with mitochondrial proteins from human mitochondrial proteins MitoCarta2.0 (Figure 6a, 6b)³⁵. The up-regulated mitochondrial proteins in late passage myoblasts were annotated according to GO_BP and designated as mitochondrial (dys) function and oxidative stress in P9 cells rather than in P3 (Figure 6c).

Long-term cultured bovine myoblasts showed loss of proteostasis

Loss of proteostasis and entry into senescence is a reason for a decline in muscle stem-cell regenerative function¹³. Our data showed that the proteasome-mediated ubiquitin-dependent protein catabolic process (by GO_BP and by KEGG) was enriched in p3 cells (Figure 5a, 5b). The proteostasis genes¹³ showed overlap (39 genes) with cluster 2 proteins (Figure 7a). The heatmap showed a down-regulation of those proteostasis proteins with increasing passage (Figure 7a, 7b). The down-regulation of proteostasis may cause defective autophagy, which disrupts satellite cell homeostasis during aging¹³.

Figure 5. Signaling pathways enriched in Cluster 2

DAVID Analysis demonstrates down-regulated signaling pathways in bovine myoblast cells from passage 9, as compared with passage 3 using gene ontology biological process annotation (GO_BP) enrichment and KEGG pathway enrichment. (a) GO_BP analysis of 443 enriched proteins in cluster 2. (b) KEGG pathway analysis of 443 enriched proteins in cluster 2. Signaling pathways were ranked on the basis of Benjamini-Hochberg corrected p-value. Each dot represents a signal pathway. Specific pathways related to satellite cell function are highlighted in red.

Figure 6. Long-term cultured bovine myoblasts showed alert mitochondrial proteins expression

(a) Area proportional Venn diagram representing 48 common genes between the up-regulated proteins in p9 and genes from the human mitochondrial transcriptome. (b) Heatmap of 48 quantified proteins expression profile in 4 biology replicates from (a). (c) DAVID analysis demonstrated signaling pathways in proteins from (a) using GO_BP enrichment. The top 10 signaling pathways were ranked on the basis of Benjamini-Hochberg corrected p-value.

Figure 7. Long-term cultured bovine myoblasts showed loss of proteostasis

(a) Heatmap of 39 quantified proteins expression profile from overlap (39 genes) between the proteostasis genes¹³ and cluster 2 proteins in 4 biology replicates. (b) DAVID analysis demonstrates signaling pathways in proteins from (a) using gene ontology biological process annotation (GO_BP) enrichment. Top 10 signaling pathways were ranked on the basis of Benjamini-Hochberg corrected p-value.

Discussion

Large scale culture of muscle stem cells is required to gain enough cells for clinical applications as well as for culturing meat. However, in muscle stem cells or myoblasts, growth arrest was shown in long-term culture^{27,28,36,37}. In addition, the differentiation ability is diminished after culturing, even after a 3 days culture^{24,28,34,38,39}. In accordance, we observed phenotypic changes in bovine myoblasts such as increased cell size as a function of passage (Figure 1) and reduced proliferation and differentiation ability (Figure 1).

Using proteomic analysis, we identified pathways that increased or decreased during long-term passage. Many of these signalings such as mitochondrial electron transport, NADH to ubiquinone^{40,41}, response to endoplasmic reticulum stress^{42,43}, cell redox homeostasis, oxidative phosphorylation, PI3K-Akt signaling pathway⁴⁴ and insulin signaling pathway⁴⁵ were up-regulated in the late passage indicating that induced oxidative stress by for instance increased reactive oxygen species (ROS) is increased with long-term passaging. The decreased expressions of DNA repair, proteostasis, fibroblast growth factor receptor¹², p53 regulation⁶ and telomere maintenance^{29,46} suggest that these signals may play a role in the loss of stemness. Those results indicate that primary bovine myoblast cells in vitro undergo a process resembling, if not being identical to, cell aging.

During aging, stem cells accumulate toxic metabolites (ROS), DNA damage, epigenetic alterations, aggregates of damaged proteins and dysfunctional mitochondria, all of which contribute to cell senescence^{2,8,47}. In our results, “mitochondrial electron transport NADH to ubiquinone”, “occurrence of an oxidation-reduction process”, “a response to endoplasmic reticulum stress”, “cell redox homeostasis”, “mitochondrial respiratory chain complex I assembly” and “oxidative phosphorylation” pathways were accrued in the **late passage** suggesting an excess of reactive oxygen species^{42,43}. Increased levels of ROS and stress signaling disrupt the genome and epigenetic status^{1,8}.

Increased “DNA repair”, “DNA damage response”, “DNA recombination”, “DNA replication” and “Mismatch repair” in the **early passage** indicate that these cells have a better functioning DNA repair system to insure genomic stability. Up-regulated “nucleosome assembly” and “histone exchange” may stabilize the epigenetic status in the **early passage**⁴⁸.

Aged stem cells also gradually lose hallmarks such as telomere length^{29,49}. In satellite cells, passaging of cells in vitro quickly and dramatically downregulates telomerase activity while aging in vivo showed a much slower rate^{29,46}. In our results, the “telomere maintenance via recombination” as a biological process, is highly expressed in **early passage**, suggesting that there is disproportional telomere shortening during in vitro culture after this initial proliferation stage has passed. The observation that in late passage there is reduced telomere maintenance via recombination confirms that

long-term cell culture is in essence a replicative senescence in bovine myoblast cells. We found that the muscle stem cells aging signal “fibroblast growth factor receptor signaling pathway” was highly expressed in the **early passage**. FGF receptor signaling is aberrant in aging satellite cells and constitutive FGFR1 signaling rescues self-renewal in SCs from aged mice¹⁴. Furthermore, augmented “Extracellular matrix organization”, “Cell-matrix adhesion” “Collagen fibril organization” in the **late passage** condition suggests that long-term culture is associated with fibrosis, which is similar in for instance WNT-induced aging of muscle stem cell¹⁸.

A recent study identified that impaired Notch signaling leads to a decrease in p53 activity, whereas Notch stimulation by recombinant Delta-like ligand 1 and subsequent enhancement of p53 promoted the survival of aged MuSCs⁶. In replicative senescence, the DNA damage signaling cascade in response to telomere erosion induces p53 activation, which in turn leads to p21 and Akt signaling^{50,51}. In our results, “regulation of signal transduction by p53 class mediator” signal was up-regulated in the **early passage**, whereas telomere shortening was still protected. Therefore, further studies on p53 and aging in vivo and in vitro are needed to clarify this relationship.

Our proteomics data shows systematic biological changes during long-term passage. The dysfunction of myoblast cells during long-term passage resembles the effect of aging. Understanding the molecular pathways involved in this long-term passage deterioration of muscle stem cell function will be critical for extending the replicative capacity of muscle stem cells and developing new cultivation method for clinical and food usage.

6

Materials and Methods

Bovine muscle tissues

The bovine satellite cells in this study were derived from fresh muscle samples obtained at a local slaughterhouse. The transport time is about 30 min.

Bovine satellite cells isolation

Bovine satellite cells were isolated from 4-6 year old male cattle as previously described and adapted to bovine tissues^{25,52,53}. The detailed method for isolation mononuclear cells from muscle tissue was described in Chapter 2.

The frozen cells were first recovered in growth medium (F10 + 20% fetal calf serum + 5ng/ml bFGF) for 2 days. The floating debris were washed away and the adherent cells were collected by trypsin dissociation. These cells were resuspended in FACS buffer and stained with APC anti-human CD29 Antibody (1:10, BioLegend, Cat# 303008), PE-CyTM7 anti-human CD56 (1:10, BD, Cat# 335826), FITC anti-sheep CD31 (1:10, BIO-

RAD, Cat# MCA1097F), FITC anti-sheep CD45 (1:10, BIO-RAD, Cat# MCA2220F) for 30-45min on ice. After antibody incubation, the cells were washed twice with cold PBS and reconstituted in growth medium. The viable CD31⁺CD45⁺CD56⁺CD29⁺ cells were isolated by cell sorting. Cell sorting was performed with a BD FACSAriaII cell sorter using 405, 488nm and 640nm lasers. Unstained cells were routinely used to define FACS gating parameters.

Satellite cell culture and differentiation

Dishes (Corning) were coated with 0.05% bovine collagen type I (Sigma, Cat# C4243). FACS isolated bovine satellite cells and unsorted cells were cultured on collagen-coated dishes in F10 medium (Gibco, Cat# 31550-023) containing 20% fetal bovine serum (FBS, Gibco, Cat# 10500-06), 5 ng/mL bFGF (R&D, Cat# 233-FB-025) and 1% P.S.. For serial expansion, cells were passaged to maintain a density of <60% confluence and counted at each passage. Bovine satellite cells differentiation was induced at 95% confluency with DMEM (Invitrogen, Cat# 41966-29) with 2% FBS. The differentiated cells were fixed with 4% PFA for immunofluorescent staining. The bright field images were acquired by AMG-EVOS microscope.

Immunofluorescent analysis of cultured myotubes.

Cultured satellite cells were fixed with ice-cold 4% PFA (in PBS) for 20 min, rinsed with PBS, and permeabilized in 0.5% Triton X-100 (in PBS) for 15 min. Permeabilized cells were blocked and incubated with primary antibodies in 1% BSA (in PBS) overnight at 4°C. Primary antibodies recognizing MyHC (1:500, Millipore, CAT# 05-716). After washing with PBS, cells were incubated with Alexa 488 labeled anti-rabbit (1:600, Invitrogen, Cat # A-11034), antibodies for 1 h at room temperature and mounted with VECTASHIELD mounting medium with DAPI (Vector Laboratories Cat# H-1500).

Differentiation efficiency percentage measurement

The percentage of MyHC⁺ nuclei per condition was expressed as MyHC⁺ nuclei divided by total nuclei in the same field. More than 1000 nuclei from 4 or more randomly chosen fields were analysed.

General, protein digestion for LC-MS analysis

Proteins from passage 3, 6, 9 were collected and digested for Liquid Chromatography – Mass Spectrometry according to the standard protocol. Protein samples were reduced with 20mM Dithiothreitol (DTT) for 45 minutes and alkylated with 40mM Iodoacetamide (IAM) for 45 minutes in the darkness. The alkylation was terminated by 20mM DDT to consume any excess IAM. Digestion was performed with a mixture of LysC and Trypsin, which was added at a ratio of 1:25 (enzyme to protein). After two hours of digestion at 37°C in a water bath, the lysate was diluted with 50mM ABC to 1M

Urea and further digested at 37°C overnight. The digestion was terminated by addition of formic acid (FA) to a total of 1%.

Liquid Chromatography – Mass Spectrometry

Peptide separation was performed on a Thermo Scientific (Dionex) Ultimate 3000 Rapid Separation UHPLC system equipped with an Acclaim PepMap C18 analytical column (2 μ m, 100Å, 75 μ m x 150 mm). Peptide samples were first desalted on an online installed C18 trapping column. After desalting peptides were separated on the analytical column with a 90 minute linear gradient from 5% to 35% Acetonitrile (ACN) with 0.1% FA at 300 nL/min flow rate. The UHPLC system was coupled to a Q Exactive HF mass spectrometer (Thermo Scientific). DDA settings were as follows. Full MS scan between 375 – 1,500 m/z at resolution of 120,000 followed by MS/MS scans of the top 15 most intense ions at a resolution of 30,000.

Mass Spectrometric Raw Data Analysis

For protein identification and quantitation, the data dependent acquisition (DDA) spectra were analyzed with Proteome Discoverer (PD) version 2.2. Within the PD software, the search engine Sequest was used with the SwissProt [*Homo sapiens*] database (*Homo sapiens* (SwissProt TaxID= 9606)). The database search was performed with the following settings. Enzyme was trypsin, a maximum of 2 missed cleavages, minimum peptide length of 6, precursor mass tolerance of 10 ppm, fragment mass tolerance of 0.02 Da, dynamic modifications of methionine oxidation and protein N-terminus acetylation, static modification of cysteine carbamidomethylation.

Mass spectrometry data processing, statistical analysis and bioinformatic analyses

The identified proteins were filtered with “Master is equal to Master”, “Protein FDR Confidence has level high in Protein FDR Confidence Combined”, “Abundances (Grouped) Counts is greater than or equal to 3 in sample group 3, 6, 9” to get all 1874 quantified proteins. For proteins up-regulated in passage 9 compared with passage 3, The total proteins were further filtered with “Abundance ratios is greater than 1.2 in ratio passage 9/ passage 3”. For proteins down-regulated in passage 9 compared with passage 3, The total proteins were further filtered with “Abundance ratios is less than 0.8 in ratio passage 9/ passage 3”. Gene ontology (GO) and KEGG pathway was performed by the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources online tool^{54,55}. Heatmaps were drawn with R software according to the results from PD 2.2. Area proportional Venn diagram was drawn according to online tool⁵⁶.

Statistics

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software). For comparisons of two treatment groups, a Student's t-test was used. For more than two groups, One-way ANOVA with Dunnett's Multiple Comparison Test. Results were means $\pm$ S.E.M. unless otherwise stated. $P < 0.05$ was considered significant.

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Chapter 7

**General discussion, future
perspectives and conclusion**

General discussion

1. Characterize and isolate high purity satellite cells in livestock animals

Maintenance of stemness in a stem cell population is of considerable scientific and societal importance. A prerequisite for maintaining stemness is to start with a highly purified stem cell population, since most other cells in culture grow faster than the stem cells, so that the latter will eventually be outnumbered¹⁻⁴. Satellite cells are a population of small mononucleated muscle stem cells in muscle tissue^{5,6}. They are the most ideal cell source for cultured meat. In mouse muscle tissue, murine skeletal muscle-derived stem cells (MDSCs) contain fibroblast-like, myogenic, endothelial, myoendothelial, pericytes and other cell populations^{7,8}. The multilineage differentiation capacity was also found in pig and human muscle derived stem cells⁹⁻¹¹. The pre-plating method used for pig and bovine obtains a various purity of satellite cells¹²⁻¹⁵. Thus, it is necessary to develop a more efficient method to obtain highly purified satellite cells in livestock for cultured meat.

Here, we performed immunofluorescent staining assays and found that both CD56 and CD29 co-localized in the same cell with Pax7 in porcine skeletal muscles. Similar results were found in bovine muscle fibers. We improved the purity of pig and bovine satellite cells by FACS and established that a combination of negative selection for CD31 and CD45 with a positive selection for CD29 and CD56 results in a high purity satellite cells population as judged by their degree of PAX7 positivity. These purified satellite cells showed high myotube formation abilities in vitro and porcine satellite cells participated in the muscle regeneration in recipient mice (Chapter 2 and 3).

Previous studies in mouse have suggested that highly purified satellite cells can be obtained by FACS sorting^{3,16,17}. The cell surface markers for FACS sorting have been well characterized in murine muscle tissues. The most commonly used cell surface markers in murine are CD34 and α 7-integrin^{1,16,17}. Recently, the CD56, CD29, epidermal growth factor receptor (EGFR) are identified as human satellite cell surface markers^{2,18}. Expression of these markers is species specific. For instance, mouse satellite cells express CD34, whereas human satellite cells do not^{2,11,16}. Our results showed that CD56 and CD29 are satellite cell surface markers for large animals (pig and bovine), which indicates satellite cells in large animals are similar with human. However, Further characterization of CD34 expression in pig and bovine satellite cells are needed.

Next generation of cell sorting can be developed from specific cell metabolism. It has been reported that glucose-depleted culture medium supplemented with lactate can facilitate purification of human pluripotent stem cells (hPSCs)-derived cardiomyocytes¹⁹. Further study found that use fatty acid as a lactate replacement together with 3,3',5-Triiodo-L-thyronine (T_3) can facilitate purification and show better function of hPSC-derived cardiomyocytes²⁰. A recent report in cancer field found that monosaccharide mannose causes growth retardation in several tumor types in vitro. Mannose also inhibited tumor growth in mice without affect the weight of the

mice, nor did it visibly affect their health²¹. These results indicate that the metabolic pathways can be used for specific cell purification or depletion. Further studies can focus on the satellite cell specific cell metabolic pathway to develop a faster, easier and safer sorting strategy for cultured meat production.

2. Mediator pathways during differentiation and long-term culture

Satellite cells are about 2-10% of total myonuclei^{17,22}. For regenerative medicine and cultured meat purpose, it is a challenge to culture large amount of functional myoblast cells. After isolation from muscle tissue, satellite cells easily start to proliferate and differentiate to Myog⁺ myoblasts^{2,18,23,24}. This differentiation is associated with less proliferative potential and lower engraftment efficiency^{23,25-28}. Several signaling pathways have been confirmed to relate to the automatic myoblast differentiation process such as p38-MAPK²⁶, JAK2-STAT3²⁵, canonical-Wnt²⁷ and extracellular laminin²⁹. By inhibiting these signals or recapitulating laminin, satellite cell function can be restored or maintained for a longer time^{25-27,29}. We studied p38-MAPK and JAK2-STAT3 function in the differentiation process. These two signaling pathways were up-regulated during differentiation. Pharmacological inhibition of p38-MAPK and JAK2-STAT3 signals inhibited the fusion process (Chapter 3 and 4).

Subculture of stem cells decreases the proliferation and differentiation abilities, especially in mesenchymal stem cells³⁰⁻³³. The defects of serial cultivation were also observed in myoblast cells³⁴⁻³⁶. We also investigated the p38-MAPK and JAK2-STAT3 signaling pathways in long-term cultured myoblast cells and they showed different effects. Inhibition of p38-MAPK maintained stemness of long-term cultured myoblast cells (Chapter 3). Pharmacological inhibition of JAK2 and STAT3 however failed to affect proliferation or differentiation of the bovine myoblast cells in long-term culture (Chapter 4). We also pharmacologically inhibited Setd7 by PFI-2, which inhibits canonical-WNT signaling²⁷ and this did not result in maintaining proliferation of bovine myoblast cells during long-term culture either (Chapter 5). These results indicate that long-term culturing of myoblast cells can be associated with either preserved or diminished differentiation capacity.

We then checked ERK and Akt signal pathways changes in differentiation and long-term culture process by Western Blot. Both ERK and Akt signals were down-regulated when the cells are confluent and then increased during differentiation. But ERK signal kept decreasing while Akt signal increased during long-term culture (Chapter 5). Those data again suggest that long-term culture may lead to different pathways and outcomes with respect to proliferation and differentiation. The expression profiles of p38-MARK, ERK and AKT signal indicate long-term culture defects that may due to in vitro aging or replicate senescence.

3. Long-term culture of bovine myoblast cells resembles the effect of aging

The long-term culture-induced limited replicative life-span of human fibroblast was firstly described in 1961 and further developed as the replicative senescence³⁷⁻³⁹. It has also been called in vitro aging of cells since replicative senescence showed similarities with in vivo aging⁴⁰. Long-term culture of myoblast cells not only showed proliferation arrest^{2,17,36,41}, but also impaired differentiation ability³⁴.

Aged satellite cells show decreased cell number, impaired self-renewal and loss of regenerative ability (when implanted in vivo in muscle defects)^{6,42,43}. The changes in muscle extrinsic tissue microenvironment function and intrinsic alterations lead to dysfunction of SCs^{6,22,43}. Several intracellular processes and signaling pathways have been described as being dysregulated in association with aging of satellite cells in vivo such as mitochondrial dysfunction^{44,45}, FGF2 and FGF receptor 1 (FGFr1) function⁴⁶, protein homeostasis (proteostasis)⁴⁷, p38^{26,46}, STAT3^{25,48}, p16⁴⁹, canonical Wnt⁵⁰, Notch signaling⁵¹, and epigenetic status^{43,52}.

We used label-free high-throughput proteomics to identify the proteins changes in early and late passages. Further gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis helped us understand the long-term culture process systematically. These signaling changes correlate with the reported aging signal of MuSCs in vivo and replicative senescence signals in vitro.

In the early passage, myoblast cells showed enriched in DNA replication, protein folding, translational initiation, DNA repair, fibroblast growth factor receptor signaling pathway, p53 regulation, proteasome-ubiquitin-dependent proteolysis, telomere maintenance via recombination and somatic stem cell population maintenance (Chapter 6). Targeting these signals may help improve the myoblast cell function during long-term culture. In aged satellite cells, FGFR1 signaling is reduced compared to those from young mice⁴⁶. Constitutive FGFR1 signaling partially rescues self-renewal in SCs from aged mice⁴⁶. However, aberrant increased FGFR signaling is also found in solid tumor types⁵³. Future studies may focus on rescuing these signals in a proper level to maintain the function of long-term cultured myoblast cells.

In the late passage, redox related signals like mitochondrial electron transport, NADH to ubiquinone^{54,55}, response to endoplasmic reticulum stress^{56,57}, Cell redox homeostasis, Oxidative phosphorylation, PI3K-Akt signaling pathway⁵⁸, Insulin signaling pathway⁵⁹ were up-regulated. This indicated that the oxidative stress might be one of the reasons for the defects during long-term passage. Indeed, slowing down or removing this oxidative stress may help in maintain geriatric satellite cell proliferation and regenerative abilities⁴⁷. p38-MAPK signaling is a stress-induced signal^{43,60}. Genetic or pharmacological inhibition of p38-MAPK signaling ameliorates age-associated satellite cell defects⁴⁶. Inhibition of p38 inhibition also helped long-term culture of human satellite cells². We investigated bovine satellite cells and showed up-regulated p38-MAPK signaling accompanied by a loss of differentiation ability during long-term

culturing in vitro. Long-term cultivation with p38i helped maintained the stemness and differentiation abilities (chapter 3). Future studies may focus on inhibiting those signals in late passages to maintain the function of long-term cultured myoblast cells.

Future perspectives for cultured meat

Meat is one of the most important dietary components for humans. However, traditional animal agriculture has a substantial contribution to climate change and air pollution, to land, soil and water degradation and to the reduction of biodiversity⁶¹. The predicted increase in demand for meat and other animal products makes the traditional animal agriculture especially become a big issue for the environment^{62,63}. Cultured meat is a technology, which potentially can solve part of the major upcoming problems with traditional livestock⁶⁴⁻⁶⁶. In this thesis, we have aimed to develop basic research to promote the development of cultured meat technology.

1. Purification of muscle stem cells

Muscle tissue is about 50-60% of the carcass and this is the most important part for raw meat and meat processing⁶⁷. Skeletal muscle tissue consists of approximately 90% muscle fibers and 10% connective and fat⁶⁸. The most important work for cultured meat is to rebuild muscle fibers. Satellite cells are an ideal cell source to produce cultured meat^{63,69}. Here, we isolated high purified pig and bovine satellite cells (Chapter 2 and Chapter 3). The MyHC⁺ myotube formation ability is more than 80% in early passages.

The purified livestock satellite cell is fundamental for cultured meat research. On the one hand, cultured meat is based on in vitro culture of satellite cells becoming myoblasts. However, there are more than 10 different cell types in muscle tissue^{7,8}. The purified livestock satellite cells can be used to study bona fide satellite cell features during in vitro culture. On the other hand, the purification of satellite cells can also help to adapt the strategy for isolation other cell types in muscle tissue⁷⁰, which may be needed to reconstruct muscle tissue with multipole cell types⁷¹. These will likely require modifications of the purification protocol.

2. Exploration of signaling pathways during myoblast cell culture

We studied several signaling pathways during satellite cell activation, proliferation and differentiation. By utilizing these signaling pathway, we can specifically control the satellite cell fate²⁴ to efficiently produce cultured meat. Upon muscle insult, quiescent satellite cells are exposed to extrinsic, pro-myogenic stimuli which activate intrinsic pathways that stimulate activation, proliferation and differentiation^{5,24}. Our data show that bovine satellite cells up-regulated p38-MAP and JAK2-STAT3 signaling during activation. ERK signal was important for subculture of myoblast cells. During

differentiation, both p38-MAPK and JAK2-STAT3 signals were essential for the myoblast differentiation. The ERK and AKT signal down-regulated at the early differentiation stage and were important for late differentiation stage (chapter 3, 4, 5).

Several growth factors can stimulate these signal pathways for myoblast cells²⁴. For example, basic fibroblast growth factor (bFGF) can stimulate p38-MAPK, ERK-MAPK, PI3-kinase/Akt, activation of STAT and stimulation of phospholipase C gamma/protein kinase C signaling⁷². Insulin-like growth factor-1 (IGF-1) stimulates ERK, Akt, STAT3 signaling⁷³⁻⁷⁵. There are several inhibitors that can inhibited unique signal pathways. The SB203580, SB 202190 are inhibitors for p38-MAPK signals^{26,46}. Due to the harmful effects, there are also some peptide inhibitors such as STAT3 inhibitor peptide aptamers⁷⁶. Future studies should focus on two issues. Firstly, the function of signal pathways during satellite cell activation, proliferation and differentiation, should be further explored. Secondly, interventions in these pathways should be evaluated, keeping in mind at stimuli or inhibitors are compatible with eventually products that are sold in the food industry.

3. Act on proteomic profiles

For the 85 g of the world first cultured hamburger, we used 10,000 muscle strips. For one muscle fiber/strip about 1.5 million myoblast cells were differentiated, which amounts to 15 billion myoblast cells to form a hamburger⁶³. Thus, the production scale of myoblasts much be very large, requiring long-term culture. The induced proliferation and differentiation defects during long-term culture of myoblast cells therefore presents an obstacle. Our proteomics data showed systematic changes in myoblast cells between early and late passage. Inhibiting or rescuing these signals may help maintaining the function of long-term cultured myoblast cells.

As an example, in our results, we have found that long-term cultivation with p38i helped myoblast cells maintaining the stemness and differentiation abilities (chapter 3). Before knowing the proteomics data, we surmised that p38 inhibition would function to maintain PAX7 expression leading to short term inhibition of differentiation. The proteomics data highlighted oxidative stress as one of the potential defects during long-term passage and indeed p38-MAPK signal is a stress-induced signal^{43,77}. This suggests that the inhibition of oxidative stress signal by p38-MAPK signaling may also be a reason for maintainece of stemness during subculture of myoblast cells^{43,77}. Future studies may focus on inhibiting or rescuing aging signals in late passage to manage large scale myoblast cells for cultured meat, for instance by additional measures to reduce oxidative stress.

Conclusion

FACS purification of bovine and pig satellite cells using CD29 and CD56 as positive markers increase the stem cell population. After long-term in vitro culturing, the satellite cells lose their stem cell identities and differentiation potentials. The defects of subculture satellite cells are not due to myoblast differentiation into myotubes as several main signaling pathways are differently expressed in the two different biological processes. Preventing the cell to differentiate in early stage myocytes/myoblasts is not sufficient to maintain the stemness of satellite cells during long-term passaging. Long-term culture of myoblast cells resembles the effect of aging. Inhibiting or rescuing aging signals may help to bring large scale bovine muscle cell culture for cultured beef applications closer to reality.

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Summary

Meat is important for a balanced human food system. It provides the macronutrients, micronutrients and lots of key elements in the diet of man, which is essential for good health. Meat demand is predicted to substantially increase due to the global population growth and meat consumption increase in many developing countries. However, the traditional animal agriculture is not sustainable. The expansion of livestock production puts pressure on natural resources, animal welfare, human and livestock health. Cultured meat is produced by obtaining stem cells from the donor animals to culture muscle tissue in vitro for meat consumption. It is a technology, which potentially can solve major upcoming problems by traditional livestock.

Satellite cells (muscle stem cells) are one of the promising cell sources for cultured meat production. However, the major focus of satellite cell research to date has been on human and mouse cells for regenerative medicine. The lack of high purity livestock satellite cells from farm animals limit the research on cultured meat. In this thesis, we characterized and isolated highly purified satellite cells from pigs and cows. In these highly purified cells, we found that long-term in vitro cultivation induced defects in proliferation and differentiation. To understand the phenomenon, we checked several important mediator pathways during differentiation and in vitro aging. Results indicate that long-term culture of satellite cells shares features with in vitro cell aging. To systematically investigate the reasons for loss of function of long-term cultured myoblast cells, we performed high throughput label-free quantitative proteomics. Bioinformatics analysis confirmed that the subculture of myoblast cells resembles the effect of aging.

In **chapter 2**, we characterized pig muscle satellite cells and provided basic information for further utilization of these cells. We cloned the full-length cDNA sequence of porcine PAX7 and characterized the numbers of satellite cells located in various pieces of skeletal muscle. A fluorescence-activated cell sorting (FACS) based method was established to isolate highly purified pig satellite cells. Using this method, we isolated pig satellite cells and found that they lost their stem cell morphology, differentiation potential, and ability to repair muscle injury in vivo after prolonged in vitro culturing.

Chapter 3, reports on a study where we set up a FACS strategy to enrich bovine satellite cells. We found that p38-MAPK signaling is activated and PAX7 expression is gradually lost during satellite cell proliferation. The p38 inhibitor (SB203580) treatment maintained PAX7 expression but inhibited the fusion of satellite cells in a concentration-dependent way in short-term incubation. The mechanism of p38 inhibition was confirmed by inhibiting canonical p38 signaling, i.e. HSP27. Long-term culture with an appropriate concentration of p38i enhanced the proliferation and PAX7 expression, while the differentiation capacity recovered and was enhanced compared to vehicle control. These studies indicate that bovine satellite (stem) cell maintenance depends on cell purity and p38 MAPK signaling. Inhibition of p38 MAPK signaling is a promising strategy to facilitate large scale cell expansion of primary cells for tissue engineering and cultured meat purposes.

In **chapter 4**, we showed that STAT3 signal was activated in cultured bovine satellite cells. Inhibition of JAK2 (JAK2i) or STAT3 (STAT3i) treatment inhibited instead of promoted myoblast cells proliferation. In accordance, JAK2i and STAT3i did not affect the expression of the stemness gene *PAX7* in bovine myoblast cells and inhibited the differentiation process. In long-term culture of bovine satellite cells, pharmacological inhibition of JAK2 and STAT3 at optimal concentrations also did not affect proliferation, stemness or differentiation. Further western blotting indicated that the JAK2-STAT3 signal is not the reason for the long-term culture defects in bovine satellite cells.

We showed several signal pathways changes in long-term culturing (as a model for aging) and in differentiation by Western blot in **chapter 5**. The two processes showed similar expression profiles in p38-MAPK signaling. The JAK2-STAT3 signal pathway was up-regulated during differentiation but remained stable in long-term culture. Both ERK and Akt signal were down-regulated when the cells were confluent and then increased during differentiation. However, ERK signal kept decreasing whereas Akt signal remained increased during long-term culture.

In **chapter 6**, we used label-free high-throughput proteomics to identify the proteins changes in early and late passages. Further gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analyses showed that proteins up-regulated in *late passage* are involved in cell-cell adhesion, muscle contraction, mitochondrial electron transport, NADH to ubiquinone and cell redox homeostasis. *Early passages* showed high expression of proteins involved in DNA replication, protein folding, translational initiation, DNA repair, fibroblast growth factor receptor signaling pathway, p53 regulation, proteasome- ubiquitin-dependent proteolysis, telomere maintenance via recombination and somatic stem cell population maintenance. These signals correlate with the aging signal of MuSCs in vivo and with relicative senescence signals in vitro. The proteomics data indicate that the dysfunction of satellite cells during long-term passage resembles the effect of aging.

In conclusion, FACS purification of bovine and pig satellite cells using CD29 and CD56 as positive markers increase the stem cell population. After long-term in vitro culturing, the satellite cells lose their stem cell identities and potential to differentiate. The defects of subculture satellite cells are not due to myoblast differentiation into myotubes as several main signaling pathways are differently expressed in the two biological processes. Preventing the cell to differentiate in early stage myocytes/myoblasts is not sufficient to maintain the stemness of satellite cells during long-term passaging. Long-term culture of myoblast cells resembles the effect of aging. Inhibiting or rescuing aging signals may help to bring large scale bovine muscle cell culture for cultured beef applications closer to reality.

Samenvatting

Vlees is belangrijk in het dieet van een mens, het biedt de macronutriënten, micronutriënten en veel belangrijke elementen, die essentieel zijn voor een goede gezondheid. Er wordt voorspeld dat de vraag naar vlees zal toenemen als gevolg van de wereldwijde bevolkingsgroei en de toename van de vleesconsumptie in veel ontwikkelingslanden. De traditionele veeteelt is echter niet duurzaam. De uitbreiding van de veeteelt zorgt voor druk op natuurlijke hulpbronnen, dierenwelzijn, gezondheid van mens en dier enzovoort. Gekweekt vlees wordt geproduceerd door stamcellen van de donordieren in vitro te kweken voor spierweefsel voor vleesconsumptie. Het is een technologie die de potentie heeft om belangrijke aankomende problemen van traditionele veeteelt gedeeltelijk op te lossen.

Satellietcellen (spierstamcellen) zijn een veelbelovende cellulaire bron voor gekweekte vleesproductie. Het onderzoek van satellietcellen is tot nu toe echter vooral gericht op mens en muis ten behoeve van regeneratieve geneeskunde. Het gebrek aan varkens- of koeien-satellietcellen met een hoge zuiverheid, beperkte het onderzoek naar gekweekt vlees. In dit proefschrift hebben we sterk gezuiverde satellietcellen in varken en koe geïsoleerd en gekarakteriseerd. In deze sterk gezuiverde celpopulaties van varkens- en rundermyoblasten zagen we dat in vitro kweken leidde tot vermindering van proliferatie en differentiatie. Om te begrijpen hoe dit gebeurt, hebben we verschillende belangrijke signaaltransductie kanalen gecontroleerd tijdens differentiatie en in vitro veroudering. De resultaten toonden dat de lange termijn kweek van satellietcellen sterke overeenkomst vertoont met in vitro cel veroudering. Om dit meer systematisch te onderzoeken, hebben we ongelabelde kwantitatieve proteomics met hoge doorvoercapaciteit uitgevoerd.

In **hoofdstuk 2** hebben we satellietcellen uit varkensspier gekarakteriseerd en basisinformatie verstrekt voor verder gebruik van deze cellen. We hebben de volledige cDNA-sequentie van varkens PAX7 gekloneerd en de hoeveelheid satellietcellen die zich in verschillende skeletspieren bevinden, gekwantificeerd. Een op fluorescentie-geactiveerde cel sortering (FACS) gebaseerde methode werd gebruikt om varkens-satellietcellen van hoge zuiverheid te isoleren. We zagen dat satellietcells, die op deze manier geïsoleerd werden, na langdurig in vitro kweken, hun morfologieën vermogen om te differentiëren in spier en spierletsel in vivo te herstellen, verloren.

Hoofdstuk 3, beschrijft een onderzoek waarin we een FACS-strategie hebben opgesteld om de satellietcellen van runderen op te zuiveren. We hebben gevonden dat tijdens de proliferatie van satellietcellen, p38-MAPK-signalerings wordt geactiveerd en PAX7-expressie geleidelijk verdwijnt. Tijdens de vroege groei van satellietcellen, werd dmv een p38-remmer (P38i, SB203580), concentratieonafhankelijk de expressie van PAX7 gehandhaafd, maar werd de fusie (differentiatie) geremd. Het mechanisme van p38-remming werd bevestigd door het remmen van klassieke p38-signalerings, d.w.z. fosforylering van HSP27. Langdurige kweek met een optimale concentratie van p38i verhoogde de proliferatie en PAX7-expressie, terwijl de differentiatiecapaciteit herstelde en verbeterd was in vergelijking met vehikel controle. Deze studies geven aan

dat optimalisatie van runder satellietcel kweek afhankelijk is van celzuiverheid en o.a. van p38 MAPK-signalering. Remming van p38 MAPK-signalering is een veelbelovende strategie om grootschalige cel-expansie van primaire cellen voor tissue-engineering en kweekvlees doeleinden te bevorderen.

In **hoofdstuk 4** toonden we aan dat STAT3-signaal geactiveerd was in gekweekte runder satellietcellen. Remming van JAK2 (JAK2i) of STAT3 (STAT3i) in vroege stadia van celkweek had een remmend i.p.v. stimulerend effect op de proliferatie van myoblastcellen, het vervolg stadium van satelliet cellen. JAK2i en STAT3i hadden ook geen invloed op de PAX7-expressie van runder myoblastcellen, en remde het differentiatieproces. Ook in langdurige kweek had ge-optimaliseerde farmacologische remming van JAK2 en STAT3 geen invloed op de proliferatie, de stamcel identiteit, of differentiatie van de runder-satellietcellen. Ook Western blot geeft aan dat het JAK2-STAT3-signaal niet de reden is voor de lange termijn kweekdefecten in runder-satellietcellen.

Met behulp van Western Blot analyse toonden we verschillende veranderingen in signaaltransductie paden gedurende lange termijn kweek (als een model voor veroudering) en in differentiatie van satellietcellen **hoofdstuk 5**. De twee processen vertoonden vergelijkbare expressie profielen in p38-MAPK-signalering. De JAK2-STAT3-signaalroute was tijdens differentiatie opwaarts gereguleerd, maar bleef stabiel in een lange termijn kweek. Zowel ERK- als Akt-activatie nam af bij het bereiken van confluentie om vervolgens tijdens differentiatie toe te nemen. Tijdens langdurige kweek, bleef het ERK-signaal echter dalen, terwijl het Akt-signaal bleef stijgen.

In **hoofdstuk 6** gebruikten we ongelabelde high-throughput proteomics om de veranderingen in eiwitten in vroege en late passages van runder satellietcellen te identificeren. Verdere genontologie (GO) en Kyoto Encyclopedia of Genes and Genomes (KEGG) -pathway-analyses lieten zien dat eiwitten die in late passage omhoog gereguleerd zijn, betrokken zijn bij cel-celadhesie, spiercontractie, mitochondriaal elektronentransport, NADH naar ubiquinon en cel-redox-homeostase. Vroege passages vertoonden hoge expressie van eiwitten die betrokken zijn bij DNA-replicatie, eiwitvouwing, translationele initiatie, DNA-reparatie, fibroblast groeifactorreceptor signaleringsroute, p53-regulering, proteasoom-ubiquitine-afhankelijke proteolyse, telomere handhaving via recombinatie en populatie onderhoud van somatische stamcellen. Deze signalen correleren met het verouderingssignaal van muizen satellietcellen in vivo en met verouderingssignalen in vitro. De proteomics-gegevens wijzen erop dat de disfunctie van satellietcellen tijdens langdurige kweek vergelijkbaar is met de veroudering van cellen.

Concluderend, FACS-zuivering van runder- en varkens-satellietcellen met behulp van CD29 en CD56 als positieve markers verhoogt de stamcelpopulatie door verhoogde zuiverheid. Na langdurig in vitro kweken verliezen de satellietcellen hun stamcelidentiteit en differentiatiepotentieel. De defecten van satellietcellen

tijdens kweek zijn niet het gevolg van myoblasten differentiatie in myotubes omdat verschillende transductie paden worden geactiveerd in de twee verschillende biologische processen. Voorkomen van vroegtijdige differentiatie van satellietcellen in myocyten / myoblasten is niet voldoende om de stamcel identiteit van satellietcellen te behouden tijdens lange termijn kweek. Langdurige kweek van myoblastcellen lijkt op het effect van veroudering. Het remmen van verouderingssignalen kan helpen om een grootschalige runder-spiercelcultuur voor kweekvlees toepassingen te realiseren.

Valorization

Humans are omnivores, eating plants and animals. Meat remains important in the human diet for many consumers in both developed and developing countries. However, meat demand is predicted to increase substantially due to global population growth and meat consumption increase in many developing countries, including China, India and Brazil and South Africa.

If that prediction turns into reality, the production of meat will become an important concern as the environmental impact of meat production is high. Cultured meat is produced by obtaining stem cells from donor animals to culture muscle and fat tissue in vitro for meat consumption. It is a technology, which has the potential to partially solve major upcoming problems with traditional livestock. My thesis mainly focuses on characterization and isolation of highly purified porcine and bovine satellite cells as cell sources for cultured meat. Since satellite cells lose proliferation and differentiation ability during in vitro culture, the reasons and solutions for this loss were also assessed. In this chapter, I will discuss the social, economic, scientific relevance of the thesis.

Social and economic relevance

Expansion of livestock meat production causes problems such as deforestation and land degradation, greenhouse gas emission, fresh water use and loss of biodiversity. Animal welfare and public health are also societal concerns associated with traditional livestock. By 2020, the global market for protein meat analogues such plant-based substitutes and cultured meat, is estimated to \$46 billion (GBP£33 billion), with significant growth potential. The global cultured meat market for the normal scenario is expected to be valued at USD 15.5 Million in 2021 and is projected to grow at a CAGR of 4.0%, to reach USD 20.0 Million by 2027. The North American region is projected to account for the largest share in the global cultured meat market because of a large meat-eating population, growing investment and research on cultured meat, and the presence of cultured meat manufacturers. The markets in Europe, Middle East & Africa, South America and Asia Pacific region are also expected to grow at fastest growth rate. The growth in cultured meat market is also influenced by the presence of major players such as Mosameat (the Netherlands), Memphis Meat (United States), Just, Inc. (United States), Supermeat (Israel) Integriculture Inc. (Japan), etc. The numbers of these companies are also growing faster.

Cultured meat has social and economic benefits. Several life cycle analysis studies have estimated that cultured meat requires smaller quantities of agricultural inputs and land. Greenhouse gas emission (GHG) would be less in cultured meat compared with traditional livestock. However, the energy use would be more or less in cultured meat depending on different calculating methods. Those estimates are sometimes contradictory because industrialization of cultured meat has not occurred yet and assumptions need to be made. It is clear however that further technological development is still needed to realise the full benefits of cultured meat for society and environment.

It is prerequisite to characterize and isolate highly purified porcine and bovine satellite cells as cell sources for cultured meat. Muscle tissue has dozens of cell types. The satellite cell is the most important cell type for cultured meat since it can differentiate into muscle with high efficiency in vitro. By isolation of highly purified satellite cells, one avoids inefficient (co)culture of cells that do not have muscle formation abilities. Loss of stemness of satellite cells in culture was also investigated and we have established some solutions for maintaining stemness during culture. Maintaining stemness results in a higher yield of cells from a single biopsy and therefore reduces the number of biopsies from live animals needed. Increasing the number of cell doublings per batch also reduces the number of transfers from small culture systems to larger systems, making the whole process more resource efficient and cost-effective.

Scientific relevance

The work presented in this thesis was primarily focused on scientific understanding of pig and bovine satellite cells. We unveiled the full-length cDNA sequence of porcine Paired box 7 (PAX7), the most important satellite cell marker. We developed fluorescence-activated cell sorting (FACS) strategy for isolating highly purified satellite cells from farm animals (like pig and cow), thus promoting satellite cell research in farm animals. The cultured meat technology will also greatly benefit from the presence of highly purified satellite cells as the cell source.

Satellite cells from mouse, dog and human lose their stemness after culturing in vitro. We observed similar results in pig and bovine satellite cells. We found that interfering with the p38-MAPK signal supports long-term culture of bovine muscle stem cells. This example brings the future of culturing functional satellite in large scale closer. Western blots and especially proteomics results indicated that the subculture of myoblast cells resembles the effect of aging. The proteomics data increase understanding of the protein expression in satellite cells leading to aging upon long-term culture. This may lead to hitherto not explored intervention in this aging process.

The aging effects of long-term culture are similarly occurring in other mammalian cells, such as fibroblast cells, endothelial cells, vascular smooth muscle cells and mesenchymal stem cells. In the future, we can also draw lessons from those cell types to fully understand the aging process during long-term culture in vitro. Likewise, our results may in part be translated to aging in those cells.

Large animals (like pigs) have many advantages in modeling human diseases due to their similar anatomic and physiological features to human beings. The work in this thesis will facilitate the production of large animal muscle organoids for drug testing and for non-food tissue engineering, for instance to repair large muscle defects for medical applications. Careful characterization of the large animal skeletal muscle stem cells (satellite cells) will also facilitate the generation of large animal models of skeletal muscle disease. For example, the swine Duchenne muscular dystrophy (DMD) model

recapitulates human symptoms better than the mouse model.

Beyond the thesis

In this work we characterized and isolated highly purified satellite cells from livestock (pig and bovine) as cell sources for cultured meat. We also investigated the reasons for loss of stemness during long-term culture and solutions to prevent these defects. Future work may focus on aging pathways. Preventing aging will facilitate large scale production of satellite cells, thus allowing the development of a cultured meat product with a higher percentage of muscle proteins. These studies on satellite cells can also be translated to other cell types in meat such as adipocytes, which in turn will eventually lead to successful development of a “real steak”.

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Curriculum Vitae

About the author:

Shijie Ding was born on February 24th 1990 in Dafeng, China. In 2008, he completed his secondary school in Jiangsu Yancheng Middle School. He studied Food Science and Technology at Nanjing Agricultural University and obtained his bachelor degree in 2012. He became a master student right after completing his bachelor degree. In 2014, he became a PhD candidate. He studied spoilage organisms in sliced hams in Prof. Zhou's lab at Nanjing Agricultural University from *Sep.2012- Nov.2013*. Then he, under the advice of Guanghong Zhou, changed his research topic to cultured meat. He moved to Shanghai to study the cell biology as the Visiting Ph.D student under the supervision of Prof. Ping Hu at Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. He learned stem cell related techniques and started to isolate and culture pig muscle stem cells for cultured meat purpose during the 3 years in Shanghai. After that, he applied for the Individual Visits grants (from Royal Netherlands Academy of Arts and Sciences) and joint PhD student scholarship (from China Scholarship Council) to study cultured meat at Maastricht University under the supervision of Prof. Mark Post. In 2017, he got the opportunity to apply a PhD-position at the lab of Prof. Mark Post. His project is about maintaining the stemness of satellite cells during long-term culture for cultured meat purpose.

Publications and grants

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1. **Ding, S. J.**, Swennen, G. N. M., Messmer, T., Gagliardi, M., Molin, D. G. M., Li, C. B., Zhou, G. H., and Post, M. J. (2018) Maintaining bovine satellite cells stemness through p38 pathway. *Sci Rep-Uk* 8 (IF 2018: 4.122)
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Abstracts:

1. **Shijie Ding.** Ex Vivo Expansion of Bovine Satellite Cells. The Third International Conference on Cultured Meat, Maastricht, The Netherlands, 2017
2. **Shijie Ding.** p38 mediates differentiation of satellite cells into myoblasts. The Dutch Physiology Society meeting- 33rd Dutch Physiology Days, Landgoed ISVW, The Netherlands, 2017.
3. **Shijie Ding.** Proteomics of bovine muscle stem cells aging. The Fourth International Conference on Cultured Meat, Maastricht, The Netherlands, 2018

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