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Development and validation of an in vitro porcine skeletal muscle model

SUSAN OMUBOBA DAN-JUMBO

A thesis presented for the degree of Doctor of Philosophy
Functional Genetics and Development
The University of Edinburgh
2022
Declaration

I declare that I have composed this thesis and that the work presented here is my own, except where acknowledgment has been made in the text. All experiments were designed by me in collaboration with my supervisors Dr. F. Xavier Donadeu and Dr. Cristina Esteves. The CRISPR experiments in Chapter 4 were designed in collaboration with Dr. Melissa Jungnickel. This work has not been submitted for any other degree or professional qualification at any other University.

Susan O Dan-Jumbo

August 2022
Abstract

The projected rise in world population to 9.7 billion by 2050 will require an increased production of meat globally to meet human requirements for protein without compromising environmental and financial sustainability. Efficient increase in meat production will require an in-depth knowledge of muscle growth and development. The ability of sows to produce large numbers of piglets per litter coupled with a farrowing rate of an average of two litters per year makes the pig a highly efficient source of meat. In addition, the anatomical and physiological similarities between pig and humans makes the pig an excellent experimental model to study human physiology and disease. The availability of robust in vitro models of skeletal muscle from livestock species is critical for understanding muscle development and disease, and to make progress towards improving animal meat production. A current technical limitation in this regard is that stem/precursor cells routinely obtained from muscle are a mixed population with low proliferative potential and low differentiation efficiency in vitro thus limiting their use in large-scale studies.

This thesis aimed to isolate, purify, and characterise myogenic cells from porcine skeletal muscle. It also aimed to develop and characterise an explant-based culture model for the isolation and maintenance in vitro of muscle stem cells from pigs. Finally, with the aim to provide proof-of-concept of the usefulness of the novel in vitro model, studies were carried out to investigate the effects of β-Klotho (KLB) and its ligand, fibroblast growth factor 21 (FGF21) on adipogenic differentiation of pig muscle-derived progenitor cells using both a siRNA mediated knockdown and CRISPR/Cas9-mediated knockout of KLB.

Using fluorescent activated cell sorting (FACS), I isolated porcine myogenic cells based on the expression of CD146 and obtained CD45-/CD31-/CD146+ and CD45-/CD31-/CD146- as myogenic and non-myogenic cell fractions, respectively. Analysis by RT-qPCR revealed that CD45-/CD31-/CD146+ fraction was indeed enriched for myogenic cells showing high expression of muscle stem cell markers, PAX7 and CD56, and low expression of preadipocyte marker, PDGFRα which was highly expressed in the CD45-
In addition, when placed in myogenic differentiation media the CD45-/CD31-/CD146+ cells were able to fuse to form multinucleated myotubes with increased expression of muscle markers MYH3 and MYOG. Interestingly, no myotubes were observed in the CD45-/CD31-/CD146- fraction. On the contrary, both CD45-/CD31-/CD146+ and CD45-/CD31-/CD146- fractions were able to accumulate lipids and differentiate into adipocytes when placed in adipoigenic media. However, CD45-/CD31-/CD146- displayed a higher adipogenic capacity with significantly increased levels of expression of fatty acid binding protein, FABP4 when compared to CD45-/CD31-/CD146+ fraction.

To establish an explant-based in vitro model, muscle tissue fragments were seeded on matrigel coated cell culture dishes to stimulate migration of muscle-derived progenitor cells (MDPCs). Expression of lineage markers in MDPCs was determined with the use of RT-qPCR and flow cytometry. In addition, their ability to differentiate into myogenic and adipogenic lineages during long term culture was also tested followed by RT-qPCR analysis of relevant lineage markers. The results showed that MDPCs displayed long-term expansion in vitro, showing an average doubling time of 48 hours. The MDPCs also expressed key muscle stem cell markers, PAX 7, MYOD, MYF5 and CD56 in the early passages while the adipogenic cell markers CD105 and PDGFRα were highly expressed at the later passages. In addition, the MDPCs were able to efficiently form myotubes over several passages. Eventually, these cells lost their myogenic potential and acquired adipogenic potential following prolonged culturing.

Finally, to demonstrate the usefulness of this in vitro model for functional molecular studies in porcine muscle, I investigated the role of fibroblast growth factor 21-βKlotho (FGF21-KLB) signalling in regulating adipogenic differentiation of porcine MDPCs using both gain of function and loss of function approaches. RT-qPCR and Immunocytochemistry analysis revealed that KLB expression was upregulated in differentiating porcine MDPCs synchronous to the formation of adipocytes and upregulation of the adipocyte markers, PPARy and FABP4. Stimulation of porcine MDPCs with FGF21
increased adipogenic differentiation while siRNA mediated knockdown and CRISPR/Cas9 mediated knockout of KLB inhibited adipogenesis by porcine MDPCs indicating that FGF21-KLB signalling is a regulator of adipogenesis in pig muscle.

In conclusion, these results show that CD146 is a suitable marker for isolation of myogenic cells from pig skeletal muscle using FACS. Moreover, I describe a simple and efficient method to purify muscle stem cells from pig skeletal muscle tissue and characterise their growth and differentiation potentials. This, together with novel know-how on gene targeting using CRISPRs provides valuable information towards future research applications to understand pig skeletal muscle development and improve meat production. Moreover, the novel in vitro model I developed could have applications in the expanding field of cultured meat. Lastly, the methods developed in this thesis may be transferable to other species provided culture conditions are appropriately optimised.
Lay Abstract

The human population continues to grow and is predicted to reach 9.7 billion by 2050. Together with increasing trends in the consumption of meat, this will put extraordinary pressure on the world demands for meat. Strategies to increase meat production must consider financial and environmental sustainability. Being a multiparous species able to produce up to 40 offspring per female a year, the pig provides a potentially very efficient source of meat. A significant issue with modern high prolificacy pig breeds is the high variability in birth weights within litters, which in turn results in variable growth efficiency and carcass quality. Growth efficiency is closely related to lean body, i.e. the proportion of skeletal muscle relative to body fat. Therefore, understanding the biological drivers of muscle growth is essential for devising efficient strategies to maximise meat production in pigs. In addition, the anatomical and physiological similarities between pig and humans makes the pig a useful biological model for human diseases.

A key limitation to the development of robust in vitro models to study muscle development is that efficient protocols to isolate and maintain in culture muscle stem/precursor cells from pig are lacking thus precluding meaningful studies. This thesis aimed to develop a protocol to isolate myogenic cells from porcine skeletal muscle samples and to establish a pig skeletal muscle cell culture system that could be used for functional gene studies.

To isolate myogenic cells from pig skeletal muscle, I used fluorescent activated cell sorting (FACS) to positively select cells that express the surface marker CD146. CD146 has been shown to mark myogenic cells in humans and mouse. The results showed that myogenic cells (CD146+) obtained by FACS expressed muscle stem cell markers; PAX7 and CD56 while non-myogenic cells (CD146-) expressed the adipogenic precursor cell marker PDGFRα. In addition, CD146+ but not CD146- cells were able to fuse and form myotubes when induced to differentiate in culture. In contrast, both CD146+ and CD146- cell fractions accumulated lipids and formed fat (adipogenesis) in culture. However, the CD146- cells displayed higher capacity to form fat compared to
the CD146+ cells, which was confirmed by the increased expression of fatty acid carrier protein (FABP4) by differentiated CD146- cells.

To establish a robust model of *in vitro* myogenesis, muscle tissue fragments were seeded on matrigel coated cell culture dishes and cells stimulated to migrate from the tissue. These cells were termed muscle-derived progenitor cells (MDPCs) and could be expanded for at least 14 passages in culture. Also, they expressed muscle stem cell markers *PAX 7, MYOD, MYF5* and *CD56*, which levels decreased with time in culture, and fat cell markers *CD105* and *PDGFRα* which levels increased at higher passages. Consistent with this, MDPCs could fused to form myotubes with high efficiently at lower passages and, as their ability to produce myotubes decreased with time in culture, they acquired an increased capacity to form fat.

Lastly, using this novel *in vitro* model of myogenesis in pig, I investigated the role of the fibroblast growth factor 21-βKlotho (FGF21-KLB) pathway on the ability of porcine MDPCs to form fat in culture. The results showed that although βKlotho (KLB) expression is barely detectable in proliferating MDPCs, it increased in differentiating MDPCs. Also, when the cells were stimulated with fibroblast growth factor 21 (FGF21), their adipogenic capacity increased but when the expression of KLB was inhibited temporarily with short interfering RNA or permanently with a genome editing tool called CRISPR/Cas9, their capacity to produce fat *in vitro* decreased. This suggests that FGF21-KLB system is important for adipogenesis of muscle cells.

In summary, my studies demonstrated that CD146 marks a myogenic cell population in pig skeletal muscle. They also report on the development of a novel and simple model of porcine myogenesis that can be used to study muscle biology in culture towards making improvements in traditional pork production as well as cultured meat. Lastly, through demonstrating the involvement of FGF21-KLB in fat production by porcine muscle cells I provided proof-of-concept of the potential of the novel model for understanding muscle biology in pigs, in addition to providing novel know-how on the use of CRISPR technology for functional gene studies with pig muscle cells.
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First and foremost, I want to thank the Almighty God for providing me with the opportunity, resources, life, and motivation to reach this significant milestone in my life.

I would like to express my sincere gratitude to my primary supervisor, Dr. Xavier Donadeu for the opportunity to undertake and complete this PhD studentship under his guidance and mentorship. His support, motivation, encouragement, and immense knowledge helped me get through this phase.

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This thesis is dedicated to you Menorah and Chara. And because I did, you too can.
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# Abbreviation

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<th>Description</th>
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<tbody>
<tr>
<td>18S</td>
<td>18S Ribosomal</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ARHGAP6</td>
<td>Rho GTPase activating protein 6</td>
</tr>
<tr>
<td>BAT</td>
<td>Brown adipose tissue</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BMP2</td>
<td>Bone Morphogenetic Protein 2</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CACCT</td>
<td>Cytosine-cytosine-adenosine-adenosine-thymidine</td>
</tr>
<tr>
<td>CAM</td>
<td>Chorioallantoic Membrane</td>
</tr>
<tr>
<td>CDK4</td>
<td>Cyclin dependent Kinase 4</td>
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<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
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<td>CHCHD3</td>
<td>Coiled-coil-helix-coiled-coil-helix domain containing 3</td>
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<td>cMET</td>
<td>tyrosine receptor kinase c-Met</td>
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<td>CO₂</td>
<td>Carbon dioxide</td>
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<tr>
<td>CRISPR/Cas9</td>
<td>Clustered Regularly Interspaced Short Palindromic Repeats/ CRISPR-associated 9 Repeats.</td>
</tr>
<tr>
<td>C-X-C</td>
<td>Cysteine X Cysteine</td>
</tr>
<tr>
<td>CXCR4</td>
<td>Chemokine receptor</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-Diamidino-2-phenylindole</td>
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<tr>
<td>DMD</td>
<td>Duchenne Muscular Dystrophy</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DML</td>
<td>Dorsal medial lip</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate-buffered saline</td>
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<tr>
<td>DSB</td>
<td>Double stranded DNA breaks</td>
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<td>Elution buffer</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<td>Ethylenediaminetetraacetic acid</td>
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<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
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<td>eGWAS</td>
<td>Expression genome-wide association study</td>
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<td>ESCs</td>
<td>Embryonic stem cells</td>
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<td>FABP4</td>
<td>Fatty acid-binding protein 4</td>
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<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>FAO</td>
<td>Food and Agriculture Organization of the United Nations</td>
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<td>FAPs</td>
<td>Fibro-adipogenic progenitors</td>
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<td>FBS</td>
<td>Foetal bovine serum</td>
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<td>Gene ontology</td>
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<td>gRNA</td>
<td>Guide RNA</td>
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<td>GWAS</td>
<td>Genome-wide association study</td>
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<td>HDR</td>
<td>Homology-directed repair</td>
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<td>Homeobox A Cluster</td>
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<td>hTERT</td>
<td>Human telomerase reverse transcriptase</td>
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<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
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<td>ICE</td>
<td>Inference of CRISPR Editing</td>
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<tr>
<td>IDT</td>
<td>Integrated DNA Technologies, Inc.</td>
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<td>IGF2</td>
<td>Insulin like Growth Factor 2</td>
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<td>immunoglobulin G</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>INDELs</td>
<td>Insertion/Deletion</td>
</tr>
<tr>
<td>iPSCs</td>
<td>Induced pluripotent stem cells</td>
</tr>
<tr>
<td>iTRAQ</td>
<td>Isobaric tags for relative and absolute quantitation</td>
</tr>
<tr>
<td>ITS</td>
<td>Insulin Transferrin Selenium</td>
</tr>
<tr>
<td>IUGR</td>
<td>Intrauterine Growth Restriction</td>
</tr>
<tr>
<td>KLA</td>
<td>α-Klotho</td>
</tr>
<tr>
<td>KLB</td>
<td>βKlotho</td>
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<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LB</td>
<td>Luria- Bertani</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic activated cell sorting</td>
</tr>
<tr>
<td>MC2R</td>
<td>Melanocortin 2 Receptor</td>
</tr>
<tr>
<td>MDPCs</td>
<td>Muscle-derived progenitor cells</td>
</tr>
<tr>
<td>MDSCs</td>
<td>Muscle-derived stem cells</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
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<tr>
<td>MGLL</td>
<td>Monoglyceride Lipase</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
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<td>mMPCs</td>
<td>murine muscle progenitor cells</td>
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<td>MPC</td>
<td>Myogenic precursor cells</td>
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<td>MPK</td>
<td>Mitogen activated protein kinases</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<td>MRFs</td>
<td>Myogenic regulatory factors</td>
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<td>MSCs</td>
<td>Mesenchymal stem cells</td>
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<td>Myogenic factor 5</td>
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<tr>
<td>MYHC</td>
<td>Myosin Heavy Chain</td>
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<tr>
<td>MYH3</td>
<td>Myosin heavy chain 3</td>
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<td>MYOD</td>
<td>Myoblast determination protein</td>
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<tr>
<td>MYOG</td>
<td>Myogenin</td>
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<tr>
<td>NCAM</td>
<td>Neural cell adhesion molecule</td>
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<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
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<tr>
<td>NG2</td>
<td>Neural/glial antigen 2</td>
</tr>
<tr>
<td>NGS</td>
<td>Next-generation sequencing</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
</tr>
<tr>
<td>NR3C1</td>
<td>Nuclear Receptor Subfamily 3 Group C Member 1</td>
</tr>
<tr>
<td>NTC</td>
<td>No Template Control</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimum Cutting Temperature compound</td>
</tr>
<tr>
<td>ORO</td>
<td>Oil Red O</td>
</tr>
<tr>
<td>P/S</td>
<td>Penicillin/ Streptomycin</td>
</tr>
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<td>PAM</td>
<td>Protospacer adjacent motif</td>
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<td>PAX3</td>
<td>Paired Box 3</td>
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<td>Phosphate Buffered Saline</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PDGFRα</td>
<td>Platelet-derived growth factor receptor alpha</td>
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<td>PFA</td>
<td>Paraformaldehyde</td>
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<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
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<tr>
<td>PPI</td>
<td>Protein-protein interaction</td>
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<tr>
<td>qPCR</td>
<td>Quantitative real-time PCR analysis</td>
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<td>Ribosomal protein L4</td>
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<td>SCs</td>
<td>Satellite cells</td>
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<td>SEM</td>
<td>Standard error of mean</td>
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<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
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<td>SP</td>
<td>Side population</td>
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<tr>
<td>SSC</td>
<td>Side scatter area</td>
</tr>
<tr>
<td>T7E1</td>
<td>T7 endonuclease 1</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris Base, Acetic acid and EDTA</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>TIDE</td>
<td>Tracking of Indels by Decomposition</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>--------------------------------------------</td>
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<tr>
<td>TNAP</td>
<td>Tissue nonspecific alkaline phosphatase</td>
</tr>
<tr>
<td>VLL</td>
<td>Ventral lateral lip</td>
</tr>
<tr>
<td>WAT</td>
<td>White adipose tissue</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>ZNF423</td>
<td>Zinc Finger Protein 423</td>
</tr>
<tr>
<td>μl</td>
<td>microlitre</td>
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Chapter 1  Introduction

1.1 Challenges Faced by the Pig Industry

The projected rise in the human population to 9.7 billion by 2050 will require a 50% increase in global food production and about a 70-80% increase in meat production to meet food requirements (https://www.un.org/development/desa/en/news/population/world-population-prospects-2019.html accessed 5th Feb2021) and (Alexandratos and Bruinsma, 2012). This rise in global food demand through 2050 is driven by population growth and the growing share of animal-based products is positively correlated with income growth particularly in developing countries (Bodirsky et al., 2015, Oecd, 2022). In addition, the environmental impact of livestock production continues to increase. For example, livestock production at present utilises 26% of all lands for pasture, 35% of all arable land to produce feed, 58% biomass appropriation for food, and 8% of the world’s water, while producing about 12-18% of greenhouse gas emissions. Thus, the livestock sector is a major user of natural resources with a significant influence on water quality, air quality, soil quality, global climate, as well as biodiversity, via the alteration of the biogeochemical cycles of nitrogen, phosphorus and carbon, resulting in environmental concerns (Leip et al., 2015).

Therefore, a top global priority is increasing meat production and quality to sustainably meet the protein requirements of a growing human population as well as finding ways to increase meat production with minimal climate impacts. Over the next decade, global livestock and fish output are expected to increase by 1.5% annually, with poultry accounting for more than half of this increase in meat production as a result of the sustained profitability and favourable meat-to-feed efficiency compared to other non-ruminants and to ruminants (Oecd, 2022, ING, 2020, USDA, 2022). According to the recent OECD Agriculture Statistics, almost all countries and regions have seen an increase in the
consumption of poultry meat with consumption expected to rise to 154 Mt, over the projection period making up about half of the additional meat consumed (Oecd, 2022, Saksena et al., 2018).

Pork has been a valuable source of food and its demand continues to increase (Orr Jr and Shen, 2006). Over the next ten years, it is anticipated that global pork consumption would rise to 129 Mt, making about a third of the growth in meat consumption overall (Oecd, 2022). According to the USDA (2017) and FAO (2017) data, pork accounts for 40.1% of global meat consumption, compared to 34.1% from poultry, 21.0% from beef and 4.8% from mutton and goat. Particularly, worldwide consumption of pork is highest in the EU member states where a consumption of 33kg/capita/year significantly exceeds the world average of 12kg/capita/year (Szűcs and Vida, 2017). Furthermore, according to recent predictions, worldwide pork output is predicted to rise by 17% by 2031, accounting for 38% of growth in meat production globally (Oecd, 2022).

As one of the most important economic traits in pig industry is the number of live born piglets per year, genetic selection for favourable traits such as high sow prolificacy has been utilised to increase production. Unfortunately, while this strategy has resulted in successful improvements in productivity characterised by an increase in the number of live born piglets (Rutherford et al., 2013), it has also resulted in an increase in within-litter variation of piglets as well an increase in the prevalence of piglets with low birthweight (Foxcroft et al., 2006) with economic consequences for the swine industry. First, the heterogeneity in body weights within litter is positively correlated with increased perinatal and postnatal mortality prior to weaning (Johnson et al., 1999). Secondly, it increases the cost of management associated with modern all-in-all-out production system (Quiniou et al., 2002).

The above challenges in managed selective pig breeding programs are further amplified by the low heritability of 2 important production traits; litter size and number of live born piglets (Urban Jr et al., 1966, Lund et al., 2002, Haley and Lee, 1992). As a result, progress in selective breeding as a means of improving
litter size has been slow over the past decades (Distl, 2007). There is also growing concerns on the impact of animal farming on the environment, it is therefore essential to seek alternative livestock breeding strategies that could potentially address the increasing environmental challenges such as global warming and risks of epidemics with infectious diseases.

Precise genetic selection or genetic bioengineering technologies offer new opportunities to address some of these environmental challenges and continues to attract increasing attention (Kim et al., 2020). Researchers in the fields of animal genomics and functional genomics have contributed to the generation of reliable genomic data. The genomes of major farm animals, including the pig, have been sequenced, and efforts to functionally annotate these genomes are ongoing paving way for transgenic and genome editing technologies that offer new opportunities for genetic improvement and, as a result, contribute to increased farm animal productivity (Tait-Burkard et al., 2018).

Similarly, cultured meat (also known as ‘cell-based meat’ or ‘in vitro meat’ or ‘clean meat’) is an area of cellular agriculture that aims to resolve sustainability issues associated with industrial livestock farming by utilising stem cell technology for large-scale industrial production of muscle tissue for human consumption (Post, 2012, Post et al., 2020, Slade, 2018). The discovery of stem cells coupled with advancement in ex vivo cell culture, and tissue engineering technologies have opened up the possibility of cultured meat with the first clean meat prototype produced and eaten in 2014 (Post, 2014). Skeletal muscle cells are the main focus for cultured meat production, however adipocytes, fibroblasts, chondrocytes, and endothelial cells are also required in the creation of a fully functional cultured meat product (Post et al., 2020).

1.2 Skeletal Muscle Growth and Development

Skeletal muscle is the most abundant tissue in the body, serving multiple functions including locomotion, powering breathing as well as providing
stability for the skeleton. Furthermore, skeletal muscle serves as the primary site of glucose uptake and storage, as well as an amino acid reservoir, giving it a critical role in glucose regulation, protein balance, and systemic body metabolism (Schnyder and Handschin, 2015, Legård and Pedersen, 2019, Iizuka et al., 2014).

Skeletal muscle is made up of fibres (myofibres) that are formed in a process known as myogenesis and these myofibres exhibit different metabolic and contractile properties (Bentzinger et al., 2012, Perruchot et al., 2012). Each muscle contains variable number of muscle fibres. These muscle fibres are formed by the fusion of a large number of muscle progenitor cells making them multinucleated and cylindrical structures, and serve as the functional units of muscle contraction (Bentzinger et al., 2012). Myofibres are structurally made up of actin and myosin filaments that are arranged in a series of repeated units to form the complex cytoskeleton structure called sarcomere. Sarcomeres are responsible for the ability to contract, which distinguishes skeletal muscles from other muscle types (cardiac and smooth muscle)(Schnyder and Handschin, 2015).

Myogenesis, the process of generating muscle involves several steps including muscle progenitor cell determination, migration, proliferation, differentiation, and fusion of myoblasts to form myotubes (Daczewska, 2020). Muscle growth and development is tightly controlled and is influenced by a combination of extrinsic and cell-specific stimuli (Thorsteinsdóttir et al., 2011).

Myogenesis can be divided into three distinct phases; embryonic, foetal, and adult myogenesis, with embryonic and foetal skeletal muscle development collectively referred to as prenatal muscle development (Bentzinger et al., 2012, Du et al., 2010).

### 1.2.1 Prenatal Muscle Development

Prenatal myogenesis comprises embryonic and foetal myogenesis and occurs in two phases known as primary and secondary myogenesis respectively.
In mammals, skeletal muscle development commences in the embryo, with all the skeletal muscles in the body, except some craniofacial muscles, being derived from progenitors present in the somites (Christ and Ordahl, 1995, Chal and Pourquié, 2017). Somites are the first structures in mammalian embryos consisting of several similar segments. These somites are transient mesodermal units that are formed in a cranio-caudal succession as a result of the segmentation of the paraxial mesoderm present on both sides of the neural tube (Figure 1.1) (Parker et al., 2003). Each somite differentiates rapidly along the dorsal-ventral axis with the ventral region forming the ventral sclerotome which gives rise to cartilage/tendons and bones of the vertebrae and ribs. The dorsal region on the other hand forms the dermomyotome which gives rise to the brown fat and dermis of the back as well as skeletal muscle progenitor cells (Pownall et al., 2002, Chal and Pourquié, 2017).

Some of these muscle progenitor cells extend beneath the dermomyotome, elongate and terminally differentiate into mononucleated myocytes thereby forming the primary epaxial myotome. The epaxial myotome gives rise to the muscles of the deep back (Biressi et al., 2007). Similarly, the hypaxial myotome which gives rise to the lateral trunk muscles is established from progenitor cells of ventral lateral lip of the dermomyotome. In addition, cells from the ventrolateral lip also undergo an epithelial to mesenchymal transition, delaminate from the dermomyotome and migrate ventrally to regions of presumptive muscle development in the limbs, ventral body wall, diaphragm and tongue (Parker et al., 2003). Lastly, cells originating/arising from the prechordal and pharyngeal head mesoderm give rise to the muscles of the head (Kuang et al., 2008). It is believed that the positional cues for the muscle progenitor cells arising from the somite are provided by the mesenchymal cells of the limb (Christ and Ordahl, 1995).
Subsequently, myogenesis continues through multiple waves of differentiation with progenitor cells differentiating into embryonic myoblasts, which in turn fuse rapidly to form the primary fibres corresponding to primary myogenesis. In the pig, embryonic or primary myogenesis occurs from days 35 to 55 of gestation. These primary fibres serve as template for formation of the secondary fibres. Secondary myogenesis occurs from day 55 to 90 of gestation in the pig during which foetal myogenic progenitors differentiate into foetal myoblasts that form the secondary fibres (Biressi et al., 2007, Dwyer et al., 1993, Wigmore and Stickland, 1983, Hernández-Hernández et al., 2017).

Figure 1.1 Illustration of the embryonic origin of skeletal muscle.

Located on either side of the notochord is the presomitic paraxial mesoderm lateral to the lateral mesoderm. The paraxial mesoderm segments along the dorsal–ventral axis and in a rostral to caudal direction into ball-like structures, known as somites. Morphogens from the notochord and the neural tube specify the somites to differentiate and form the dermomyotome and the sclerotome. The dermomyotome is subdivided into the hypaxial and the epaxial dermomyotome, and is the source of cells for the lateral trunk musculature and deep back musculature, respectively. The sclerotome segregates and cells from the dorsal medial lip (DML) and ventral lateral lip (VLL) migrate under the dermomyotome to form the epaxial myotome and the hypaxial myotome respectively. At the level of the limb bud, cells of the VLL also undergo an epithelial to mesenchymal transition, delaminate and migrate to regions of limb bud and later give rise to limb muscles (migrating hypaxial cells). The lateral-plate mesoderm is the source of several factors, such as the bone morphogenetic proteins (BMPs), which negatively regulate myogenic differentiation through the downregulation of MyoD expression. Image adapted from (Parker, Seale et al. 2003).
It is generally agreed that in the pig the total number of muscle fibres is definitively established by days 90-95 of gestation when secondary myogenesis is completed. Subsequently, from late gestation through the first postnatal weeks, these fibres undergo a process of maturation/hypertrophy to establish the highly organized fibre pattern seen in the adult pig (Wigmore and Stickland, 1983, Dayton and White, 2008).

1.2.2 Regulation of Prenatal Myogenesis

A group of muscle specific transcriptional factors (myogenic regulatory factors -MRFs) tightly regulate myogenesis and include myogenic factor 5- MYF5; myoblast determination protein-MYOD; muscle-specific regulatory factor 4- MRF4 and myogenin (MYOG). The MRFs regulate muscle lineage differentiation as they play essential roles in satellite cell activation, proliferation, and terminal differentiation (Cornelison and Wold, 1997, Seale and Rudnicki, 2000). Upstream of the MRFs are two members of the paired-box transcription factor family, paired box 3 (PAX3) and paired box 7 (PAX7) which are important for the specification of muscle stem cell commitment (Biressi et al., 2007, Buckingham, 2006, Buckingham and Relaix, 2007).

During embryonic development, myogenic precursor cells of the dermomyotome are marked by the expression of PAX3 (Goulding et al., 1994, Kiefer and Hauschka, 2001, Christ and Brand-Saberi, 2004) which is required for segmentation of the somite, formation of the dermomyotomal lips and coordinates multiple aspect of limb myogenesis (Relaix et al., 2004, Schubert et al., 2001). As myogenesis progresses into the foetal stages, myogenic precursor cells downregulate PAX3 and express PAX7. These PAX7+ cells support late muscle growth, take up satellite cell positions between basal lamina and sarcolemma prior to birth and constitute the satellite cell pool in adult muscle tissue (Hutcheson et al., 2009, Seale et al., 2000, Schienda et al., 2006, Comai and Tajbakhsh, 2014).
Myogenesis depends on the expression of PAX3 and PAX7 since these proteins are essential for ensuring the survival of embryonic, foetal, and adult muscle progenitors. Additionally, the expression of PAX7 and PAX3 causes the MRFs to be upregulated (Sambasivan et al., 2011, Oustanina et al., 2004, Bober et al., 1994, Relaix et al., 2004, Bailey et al., 2001). Generally, MYF5, MYOD, and MRF4 are necessary for skeletal muscle determination and the acquisition of a myoblast precursor destiny, whereas MYOG, MYOD, and MRF4 are necessary for terminal skeletal muscle differentiation (Comai and Tajbakhsh, 2014). Thus, the earliest marker of committed muscle cells is MYF5, followed by MYOD (Lepper and Fan, 2010). MRF4 functions as a determination gene and is primarily expressed in the early stages of undifferentiated proliferating cells (Kassar-Duchossoy et al., 2004). MRF4 is also later expressed in post-natal muscles and functions as the major MRF at this stage (Stewart and Rittweger, 2006). MYOG is essential for committed myoblasts to terminally differentiate and fuse (Barnoy and Kosower, 2007).

Following downregulation of PAX3 and PAX7, the early MRFs, MYF5 and MYOD are upregulated in myogenic progenitors, which then go through multiple rounds of cell division before exiting the cell cycle as MYOD expression increases. These cells then begin the terminal differentiation phase when they eventually differentiate into myoblasts expressing MYF4 and MYOG. MYOG stimulates the migration and alignment of myoblasts after mutual recognition and subsequently fusion of myoblasts to form multinucleated myofibres (Figure 1.2) (Chen and Olson, 2004, Manceau et al., 2008, Horsley and Pavlath, 2004).

Finally, the intermediate filament proteins; desmin and vimentin, as well as the sarcomeric filament proteins myosin, actin, and actinin, are structurally organised resulting in the maturation of the myofibres (or muscle cells). These proteins aid in the sarcomeric contractions and support the structure of the muscle (Jacquemin et al., 2004).
1.2.3 Postnatal Muscle Growth and Muscle Regeneration

After birth and until the prepubertal period, skeletal muscle undergoes substantial growth and maturation characterized by extensive morphological and metabolic changes (Bachman et al., 2018, Schiaffino et al., 2013). During this time, bone growth and elongation passively pull on the skeletal muscles, causing increases in muscle weight, muscle length, as well as the organisation
of the myofilament (Olwin et al., 1994, Khodabukus et al., 2018). As the number of muscle fibres is fixed around birth, postnatal muscle growth is primarily caused by an increase in muscle fibre size (hypertrophy), rather than increase in the number of muscle fibres (hyperplasia) via new myofibre formation (except during muscle regeneration) characteristic of prenatal myogenesis (Wigmore and Stickland, 1983, Rehfeldt et al., 2000, Brameld and Daniel, 2008). As a result, total fibre number is an important factor in postnatal muscle growth. The main contributors to postnatal muscle growth are the muscle stem cell population known as satellite cells (SCs) that reside adjacent to muscle fibres juxtaposed between the basal lamina and the sarcolemma (Schultz and McCormick, 1994, Yin et al., 2013, Moss and Leblond, 1971). During the postnatal period of skeletal muscle growth, these satellite cells proliferate rapidly into their progeny, myoblasts, and serve as a source of new myonuclei. When these myoblasts fuse with existing muscle fibres, they provide external nuclei that enhance the protein content and protein synthesis capacity of each fibre, leading to an increase in muscle size (Chen and Olson, 2004, Le Grand and Rudnicki, 2007).

Adult myogenesis is triggered by stimuli such as acute injury or chronic disease that disrupt the sarcolemma of the myofibres, necessitating muscle regeneration to repair the damage (Chargé and Rudnicki, 2004, Price et al., 2007, Tedesco et al., 2010). Muscle regeneration refers to this progression from myofiber necrosis to new myofiber formation. Importantly, the injured tissue must retain an extracellular matrix scaffolding that serves as a template for the formation of muscle fibres. The muscle injury causes fibre necrosis and recruitment of inflammatory cells to remove dead tissue with subsequent recruitment and activation of SCs to the site of injury to begin tissue regeneration process (Chargé and Rudnicki, 2004, Bischoff, 1994). These activated SCs proliferate and differentiate into muscle precursor cells (MPC) or myoblasts that are capable of fusing with existing myofibres to repair them or with neighbouring MPC to form new myofibres. As the activated SCs proliferate rapidly, a small portion of the daughter cells renews the original
satellite cell (SC) pool, but the majority of these cells differentiate to myoblasts (Yin et al., 2013, Tidball, 2017).

1.2.4 Muscle Fibre Types

Skeletal muscle is made up of both slow-twitch oxidative myofibers and fast-twitch glycolytic myofibers, which affect muscle metabolism, function, and,
eventually, whole-body physiology in various ways (Lee et al., 2015b). Myosin heavy chain isoforms (MYHC) were found to be the best fibre type-specific marker and are usually used for the characterization of the contractile properties of fibres (Murgia et al., 2017). On the basis of MYHC isoforms muscle fibres are categorised as Type I (slow twitch), Type IIA (fast oxidative), and Type IIB (fast glycolytic) and Type IIX (intermediate fast) fibres (Lefaucheur, 2010, Schiaffino, 2018, Schiaffino and Reggiani, 2011).

In general, Type I muscle fibres are the slowest to contract, whereas type IIB are the fastest, and type IIA and IIX are intermediate. Muscle with a higher proportion of type I and type IIA fibres are rich in myoglobin and mitochondria with characteristic slow contractile properties. These slow muscle type uses more oxidative phosphorylation as an energy source, allowing it to be active for longer periods of time (Lefaucheur, 2010, Schiaffino, 2010, Murgia et al., 2017). On the other hand, Type IIX and IIB fibres have low myoglobin and mitochondrial content, but abundant glycolytic enzymes, with fast contraction and relaxation time. These fast muscles produce more ATP through glycolytic metabolism (Schiaffino, 2018, Schiaffino and Reggiani, 2011, Murgia et al., 2017).

It is important to note that not all muscle fibres are purely one type or another, and a variety of intrinsic and extrinsic factors, such as muscle location, species, breed, genotype, gender, age, activity, ambient temperature, diet, and growth-promoting substances, may influence muscle histochemical properties (Pattanakuhar et al., 2017, Kristensen et al., 2015). Within species, genetic factors such as breed, genotype, and the presence of key genes have a considerable influence on muscle fibre type composition, and even within individuals of the same breed reared under identical nutritional and environmental conditions, fibre type composition is extremely varied (Lefaucheur, 2010).

In terms of fibre composition, the semitendinosus muscle (ST) contains a mixture of Type I (slow-twitch) and Type II (fast-twitch) muscle fibres. However, the exact fibre type composition can vary depending on factors such as
location within the muscle, age, sex, and level of physical activity (Petersen et al., 1998, Totland et al., 1988, Sen et al., 2015). Studies in pigs have shown that the ST muscle have a large proportion of Type IIB fibres and low proportion of Type I fibres (Granlund et al., 2011). In a comparative study in the pig, Realini et al. (2013b) showed a higher number of Type I fibres in masseter muscle compared to ST. However, white ST muscle had a predominance of fibre type IIB, while red ST showed a predominance of fibre Type I (Realini et al., 2013a, Gunawan et al., 2007).

### 1.3 Adipose Tissue Development in the Pig

The adipose tissue functions in energy storage and plays a critical role in the regulation of whole body metabolism and homeostasis (Yan et al., 2013). The morphology, location and physiology of the adipose tissue varies greatly among depots and type. There are two main types of adipose tissue in mammals: white (WAT) and brown (BAT) (Louveau et al., 2016). BAT is characterized by multilocular cells with high mitochondrial content and is essential for thermal regulation especially in newborn infants. WAT on the other hand contain unilocular adipocytes characterized by a central lipid droplet which primarily function as the site of triacylglycerol storage in the body (Poulos et al., 2010). Postnatally, WAT forms in a number of individual depots including in the abdominal cavity (visceral depots e.g. perirenal), under the skin (subcutaneous depots, the more abundant in pigs) and within the musculature (inter- and intra-muscular depots) (Louveau et al., 2016).

In most species, BAT is predominant around birth and in newborn infants, BAT functions mainly in thermogenesis (Oelkrug et al., 2015). However, the pig lacks BAT due to disrupted uncoupling protein 1 (UCP1) gene in the pig lineage, and in these species, neonates utilise shivering and the presence of nest littermates to cope with cold exposure (Pedersen et al., 2020, Berg et al., 2006). Moreover, the insulating role of WAT at subcutaneous location (seen abundantly in pig neonates) to participate in the maintenance of core temperature during cold exposure is well-recognized (Louveau et al., 2016).
Adipogenesis is defined as the process of proliferation, differentiation, and maturation of adipose precursor cells to form adipocytes capable of lipogenesis and lipolysis (Hausman et al., 2014b). During this process, precursor cells are devoid of lipid but become committed to the adipocyte lineage (called preadipocytes). Preadipocytes may remain quiescent, proliferate or differentiate into mature, lipid-containing adipocytes (Poulos et al., 2010). Preadipocytes are primarily derived from mesoderm-derived mesenchymal stem cells (MSCs) during embryogenesis and foetal development (Hausman, 2012). In the developing pig foetus, muscle and bone development precedes fat formation, with muscle growing relatively quickly and bone growing relatively slowly. Fat formation accelerates during the later stages of development at which time the rates of muscle and bone deposition decreases (Wagner et al., 1999, Tess et al., 1986, McMeekan, 1940). In the pig foetus, the onset of adipose tissue development is marked by the appearance of a presumptive mesenchymal fat tissue which develops into a number of “primitive fat cell organs or lobules” which in turn increase in number and size throughout foetal development (Reviewed by (Poulos et al., 2010)). The first groups of adipocytes are seen subcutaneously between 50 and 75 days of gestation thus overlapping with secondary myogenesis. Subsequently, perirenal adipose tissue develops at approximately 70 days of gestation and the first intramuscular adipocytes appear during the first month after birth (Hausman et al., 2014b).

Although foetal fat gain accelerates from 69 days post-conception onwards, at birth, pig neonates are characterized by a small amount of total body fat which is less than 10g mobilizable fat per kg body weight (Le Dividich et al., 1991). After birth, there is rapid differentiation and growth of the adipose tissue resulting in marked changes in adipose tissue structural components as well as adipocyte size (Schinckel et al., 2008, Wiseman et al., 2007). In general, the development of pig adipose tissue is attributed to cellular hyperplasia between 7 and 20 kg, to both hyperplasia and hypertrophy between 20 and 70 kg, and to cellular hypertrophy alone above this body weight. As a result, the
increase in fat deposits during pig growth is primarily caused by an increase in adipocyte size (Anderson and Kauffman, 1973).

1.4 Muscle Stem/Progenitor Cells

Stem cells in a quiescent state are found residing in most tissues of the adult body. In addition to possessing self-renewal ability, these cells are capable of differentiating into different lineages and play a role in normal tissue repair and maintenance (Dhawan and Rando, 2005, Rando, 2006). Also, adult stem cells are utilised in various medical applications such as gene therapy, tissue transplantation, the treatment of degenerative diseases, tissue engineering, enhanced tissue repair, and even the amelioration of dysfunction associated with normal ageing (Rando, 2006, Wilschut et al., 2008). In addition, increasing public awareness of foodborne illnesses, factory farming, and the ecological footprint of the meat industry has led to the utilisation of stem cells in generating cell-based meat (i.e., clean meat), a field that has gained tremendous popularity in the last decade (Ben-Arye and Levenberg, 2019, Melzener et al., 2021, Dohmen et al., 2022).

Skeletal muscle stem/progenitor cells play an important role in skeletal muscle function and regeneration, so many different types of stem cells are being studied to aid our understanding of muscle biology and stem cell behaviour and for their potential use in the treatment of skeletal muscle diseases. Practically speaking, the optimal stem cell population for treating muscle abnormalities should be found in readily accessible postnatal tissues, expandable in vitro, capable of differentiating into skeletal muscle cells in vivo, and able to enter skeletal muscle systemically (Dellavalle et al., 2007, Gois Beghini et al., 2019). Despite many identified perspectives, skeletal muscle-derived stem cells show significant limitations in regenerative studies.

Firstly, there is a low post-injection survival rate and limited migration, resulting in insufficient targeting and a moderate regeneration capacity. Higher doses of stem cells with migratory ability must be administered to overcome this
limitation. Cell expansion *in vitro* is therefore necessary to provide a higher cell number. In addition, the cell culture conditions need to be optimised to avoid loss of stemness and change in cell phenotype that would otherwise occur. With respect to cultured meat, *in vitro* expansion of muscle myogenic and adipogenic stem cell populations is essential for scalable production of cultured muscle and fat tissue (Choi et al., 2020c, Carnes and Pins, 2020, Tongers et al., 2011, Pittenger et al., 2019, Liu et al., 2018).

Secondly, several reviews of the properties of the muscle stem cell populations indicate that different stem cell populations play different roles in skeletal muscle growth and regeneration in physiologic and disease conditions. With respect to potential for the treatment of skeletal muscle injuries and diseases, varying results have been obtained with some stem/progenitor cells yielding greater skeletal muscle regenerative capacity than others (Péault et al., 2007, Mierzejewski et al., 2020a, Yin et al., 2013, Shi and Garry, 2006). Therefore, a thorough characterization of muscle stem cells will provide information for establishing stem cell cultures with high-potential for cell-based therapies.

Lastly, while studies in mouse model of human muscle myopathies has helped advance the knowledge on muscle resident stem/properties and their potential role in skeletal muscle repair and regeneration, results are not directly translated to humans because in many cases they fail to mimic the biology of affected humans due to variation in life span, physiology and genetics between mice and humans (Khodabukus et al., 2018). The pig on the other hand is anatomically and physiologically similar to human and is regarded as one of the suitable xenograft donors for humans (Holzer et al., 2005, Petersen et al., 2009). Therefore, pig muscle stem cells may be suited for xenotransplantation and serve as great biological models in translational research. Moreover, availability of robust *in vitro* models of skeletal muscle from livestock species like the pig is critical for understanding muscle development and disease, and to make progress towards improving animal meat production.
1.4.1 Satellite Cells

Satellite cells (SCs) are bona fide stem cells found in late-stage foetal and post-natal skeletal muscle, having the ability to self-renew, proliferate, and differentiate into myoblasts which fuses to form myotubes (Yin et al., 2013, Asakura et al., 2001, Partridge, 2004, Morgan and Partridge, 2020). As first identified by electron microscopy, SCs are closely associated with muscle fibres, juxtaposed between the basal lamina and fibre membrane (sarcolemma). In addition, their chromatin and organelle characteristics suggest that they are mitotically and metabolically quiescent (Mauro, 1961, Cho and Doles, 2017).

Once activated by stimuli such as weight bearing or other trauma such as injury or disease, SCs transiently proliferate to produce their progeny often referred to as myogenic precursor cells (MPCs) or myoblasts (Yin et al., 2013). These myoblasts undergo multiple rounds of cell division to become fusion-competent myoblasts capable of differentiating terminally to form multinucleated myotubes (Seale and Rudnicki, 2000, Hawke and Garry, 2001, Tidball, 2017, Yin et al., 2013). SCs function during peri- and postnatal myogenesis by contributing new nuclei to growing muscle through fusion with the adjacent fibre (Weissman, 2000, Partridge, 2004, Kaczmarek et al., 2021). Once muscle development has been completed, they become quiescence and henceforth reside within it (Dhawan and Rando, 2005, Schultz and McCormick, 1994). SCs account for approximately 2-5% of the nuclei associated with a muscle fibre and primarily function in postnatal muscle growth and regeneration of injured muscle (Cerletti et al., 2008, Tedesco et al., 2010). Postnataally, muscle growth potential is determined by the number of muscle fibres formed prenatally and the number of satellite cells in a fibre, as well as their propensity to proliferate and differentiate (Rehfeldt et al., 2000).

As stem cells, SCs undergo both symmetrical and asymmetrical cell division to replicate themselves (self-renew) and maintain tissue homeostasis as well as produce functional progeny that is able to differentiate. Cell polarity as well as the orientation of the mitotic spindle in relation to the longitudinal axis of the
myofibre determines whether a satellite cell divides symmetrically or asymmetrically (Kuang et al., 2007, Tidball, 2017).

In response to severe injury, SCs can either undergo symmetrical divisions to produce two daughter satellite myogenic cells or asymmetrically to produce one mother satellite stem cell and one daughter satellite myogenic cell (Morrison and Kimble, 2006, Sacco et al., 2008) (Figure 1.4). The mother satellite stem cell has the self-renewing capacity and replenishes the satellite cell pool while the daughter satellite myogenic cells undergo extensive cell proliferation, terminally differentiate and fuse with one another to form new myofibres or with existing damaged fibres thereby contributing to muscle repair (Yin et al., 2013, Dumont et al., 2015, Tierney and Sacco, 2016). Asymmetrical divisions predominantly occurs when the mitotic spindle is oriented perpendicular to the axis of the muscle fibre with the resulting mother SC and the daughter SC lying in close proximity to the basal lamina and the myofibre plasma membrane, respectively (Kuang et al., 2007).

SCs have different characteristics than their daughter myoblasts (Seale et al., 2000). While quiescent SCs express PAX7, PAX3 and MYF5, activated SCs express MYOD, MYOG, and MRF4 in addition to PAX7 (Asakura et al., 2001).
Adult stem cells are found in specific locations or niches that are ultimately responsible for the maintenance of stem cell populations as well as their controlled proliferation and differentiation. These niches provide stem cells specific signals and physical support in the form of specialized cells and/or extracellular matrix as well as the instructions for these cells to stay committed to their respective organ-specific cell lineages (Bentzinger et al., 2012).

SCs are found closely attached to the myofibre and covered by the extracellular matrix of the basement membrane (Mauro, 1961). Quite often, this location is in close proximity to capillaries, which supply the essential nutrients thus acting as the SC niche (Christov et al., 2007). Their niche regulates the fate of SCs, allowing them to remain arrested in a quiescent, 

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**Figure 1.4 Satellite cell symmetric and asymmetric division.**

Satellite cells can undergo symmetric divisions to expand the satellite cell or asymmetric divisions to maintain the stem cell population and generate myogenic progenitors. During asymmetric cell division satellite stem cell (purple) gives rise to one daughter PAX7+ satellite stem cell (purple) which maintains satellite stem cell pool by self-renewal and one PAX7+/MYF5+ committed satellite myogenic cell (green). Symmetric division of satellite stem cell (purple) results in two identical daughter cells (purple). Satellite myogenic cells can only undergo Symmetric division and cannot give rise to satellite stem cell. Image from Yin et al., 2013.
non-proliferative (G0) state, which is critical for their lifelong maintenance. The dependence of SCs on their niche is becoming dramatically apparent with regard to the poor success rate of stem cell therapy for diseased muscle (Mierzejewski et al., 2020a, Fukada et al., 2022, Péault et al., 2007). Disturbance of the niche, as seen during muscle injury or experimental extraction triggers activation of the SCs. Once activated, they exit G0 and enter a prolonged G1-phase (25–35 hr), undergo rapid proliferation (expressing by that time not only Pax7 but also MYOD) and differentiation during which time they lose Pax7 and express myogenin (Cornelison and Wold, 1997, Mourikis et al., 2012).

1.4.2 Other Muscle Stem/Progenitor Cell Populations

The majority of adult muscle nuclei are derived from SCs, implying that postnatal muscle growth potential is highly related to the number of SCs per muscle fibre, as well as their proliferation and differentiation (Allen and Rodman, 1979, Duddy et al., 2015). However, besides SCs, several distinct stem cell populations including pericytes, hematopoietic cells and various types of mesodermal stem cells which are not somitic in origin have the capacity to differentiate into myogenic cells and thus contribute to postnatal muscle growth (Du et al., 2013, Morgan and Partridge, 2020, Wosczyna and Rando, 2018).

Skeletal muscle contains a diverse progenitor cell population accounting for the variety of tissue types it contains including adipose, vascular and connective tissue (Zammit and Beauchamp, 2001). These populations display varying marker expression patterns, a wide range of differentiation and self-renewal potential, and most likely play various roles in skeletal muscle regeneration. For example, studies have shown that some populations are multipotent progenitors capable of differentiating into myogenic, adipogenic, chondrogenic, hematopoietic, osteogenic, and even neurogenic lineages (Asakura et al., 2001, Goodell et al., 2001, Gautam and Yao, 2019, Guimarães-Camboa et al., 2017).
1.4.2.1 Muscle Side Population

Muscle side population (SP) cells are an alternative myogenic progenitor population identified in skeletal muscle and bone marrow (Gussoni et al., 1999, Majka et al., 2003, Asakura et al., 2002a, Klimczak et al., 2018). In skeletal muscle, they reside in muscle interstitium in close proximity to blood vessels (Gussoni et al., 1999, Schienda et al., 2006, Doyle et al., 2011). Although they lack a consistent pattern of cell surface marker expression, they can be isolated by fluorescent activated cell sorting (FACS) based on their characteristic ability to efflux Hoechst 33342 DNA dye from dissociated muscle (Schienda et al., 2006, Asakura et al., 2002a, Gussoni et al., 1999, McKinney-Freeman et al., 2003).

Although lineage tracing studies showed that the bulk of muscle SP cells share a common ancestry with SCs and arise from hypaxial somite, others suggested they arise from bone marrow, endothelial or hematopoietic stem cells, a distinct origin from SCs, indicating that they are heterogenous (Schienda et al., 2006, McKinney-Freeman et al., 2003). Muscle SP cells are indeed distinct from SCs based on their anatomical location in muscle, their characteristic ability to exclude the Hoechst Dye and their presence in muscle of SC deficient mice (Pax7-/--mice) (Seale et al., 2000, Asakura et al., 2002a, Relaix et al., 2007).

In vitro studies showed that muscle SP cells were able to differentiate into myogenic cells expressing activated or quiescent satellite cell markers when co-cultured with primary myoblasts or through the forced expression of PAX7 or MYOD, indicating that they may contain SC progenitors as well as more committed myogenic progenitors (Asakura et al., 2002a, Seale et al., 2004, Uezumi et al., 2006). In addition, following intravascular and intramuscular injection into regenerating muscle, muscle SP cells demonstrated the ability to engraft skeletal muscle, replenish the myogenic stem cell pool and regenerate tissue indicating that they may contribute to long term muscle regeneration (Asakura et al., 2002a, Bachrach et al., 2004, Gussoni et al., 1999).
With respect to cell surface marker expression patterns and functional capabilities, muscle SP cells are an heterogeneous and multi-potent cells capable of differentiating into myogenic cells although they exhibit a haematopoietic lineage preference (Lapan et al., 2012a, Asakura et al., 2002a). For instance, based on the expression of CD45 (haematopoietic cell surface marker), two populations of muscle SP have been identified. While both CD45+ SP cells and CD45- SP cells show myogenic potential in vitro, the somite derived CD45- SP cells have a more myogenic potential than CD45+ SP cells (Schienda et al., 2006). Further research has revealed that the hematopoietic potential of muscle SP cells is restricted to CD45+ SP cells as they could engraft into the hematopoietic compartment of lethally irradiated recipients whereas the "myogenic" subset is CD45-negative (McKinney-Freeman et al., 2002, McKinney-Freeman et al., 2003).

1.4.2.2 Myo-endothelial Cells:

Myo-endothelial cells are a subset of SCs that are found in the vascular endothelium of human skeletal muscle (Crisan et al., 2008a, Péault et al., 2007). Flow cytometry analysis revealed that myo-endothelial cells co-express myogenic (CD56) as well as endothelial (CD34; CD144) surface markers and can be sorted as a homogenous population of CD56+/CD34+/CD144+/CD45- cells (Zheng et al., 2006). Like myogenic cells, both myo-endothelial cells (CD56+/CD34+/CD144+/CD45-) and muscle endothelial cells (CD56-/CD34+/CD144+/CD45-) were able to regenerate muscle fibres within cardiotoxin-injured mouse muscle when injected intramuscularly. However, myo-endothelial cells displayed a higher muscle regenerating potential compared to the myogenic cells suggesting that they are a good candidate for treatment of muscle diseases (Zheng et al., 2006, Tamaki et al., 2002).
1.4.2.3 Mesoangioblasts

Mesoangioblasts are mesodermal stem cells that were first isolated from the embryonic dorsal aorta of mouse characterised by the expression of several pericyte and early endothelial markers (De Angelis et al., 1999, Minasi et al., 2002, Tonlorenzi et al., 2017). In vitro studies revealed that these cells have the capacity to proliferate extensively and long-term cultured mesoangioblasts are multipotent, capable of giving rise to multiple mesodermal lineages after transplantation, including bone, cartilage, smooth, cardiac, and skeletal muscle (Minasi et al., 2002). Although the role of mesoangioblasts in normal muscle development is debatable, studies have shown that mesoangioblasts can be used to aid in muscle regeneration, particularly in dystrophic muscles. For example, Intra-arterial injection of either wildtype or genetically corrected mesoangioblasts expressing sarcoglycan, could robustly generate sarcoglycan+ myofibres and functionally improve the dystrophic phenotype in a mouse model of limb muscular dystrophy lacking sarcoglycan (Sampaolesi et al., 2003). Also, in this mouse model, enhancing the delivery of mesoangioblasts by in vitro exposure to stromal cell derived factor-1 and expression of α-4 integrin enhanced transmigration in vitro and migration into dystrophic muscle in vivo and resulted in the complete reconstruction of downstream skeletal muscles (Galvez et al., 2006).

The extensive distribution of donor cells throughout the capillary network was responsible for the high efficiency of normal myofibre reconstitution, indicating that the inherent characteristics of mesoangioblasts may enable them to travel to regenerating muscles via the bloodstream. Additionally, the local SCs in this mouse model have a natural deficiency in proliferation, giving the donor cells that were injected a selective advantage (Yín et al., 2013, Péault et al., 2007). Furthermore, mesoangioblasts have the capacity to secrete immunosuppressive and tolerogenic chemicals, which are thought to aid their integration into allogeneic dystrophic hosts (Guttinger et al., 2006). These results have revealed significant therapeutic potential of mesoangioblasts for the treatment of human dystrophic diseases.
1.4.2.4 Pericytes

Pericytes (Rouget cells or Mural cells) are contractile connective tissue cells that closely encircle capillaries and microvessels and are distinguished by the expression of CD146, NG2 proteoglycan, and platelet derived growth factor receptor (PDGFRβ) (Vezzani et al., 2016, Dellavalle et al., 2011, Armulik et al., 2011, Crisan et al., 2008b, Crisan et al., 2008a). Pericytes are multipotent cells with capability to differentiate into multiple lineages such as skeletal muscle, adipocytes, chondrocytes and osteoblasts *in vitro* (Vezzani et al., 2016, Sacchetti et al., 2016, Dellavalle et al., 2011, Crisan et al., 2008a, Dellavalle et al., 2007, Farrington-Rock et al., 2004). They lack the expression of myogenic markers (PAX7, MYF5, MYOD), hematopoietic marker (CD45) and endothelial markers (CD144, CD31, CD34) making them distinct from SCs, embryonic mesoangioblasts and hematopoietic cells respectively (Dellavalle et al., 2007). However, regardless of origin, pericytes isolated from human skeletal muscle and non-muscle tissues such as adipose, placenta, and pancreas exhibited myogenic potential *in vitro* and *in vivo* (Crisan et al., 2008b).

Intra-arterially injected human pericytes incorporated beneath the basal lamina, gave rise to numerous dystrophin-expressing muscle fibres, and enhanced the functional performance of treated dystrophic muscle in mice with combined immune deficient-X-linked muscular dystrophy (scid-mdx mice) (Dellavalle et al., 2007). Similarly, when pericytes containing a modified dystrophy gene were expanded *in vitro* prior to intraarterial injection, they gave rise to a large number of dystrophin expressing myofibres indicating a muscle-regenerating potential for pericytes (Dellavalle et al., 2007). Mouse endogenous alkaline phosphatase positive (ALP+) pericytes were able to enter the satellite cell niche and fuse with myofibres during postnatal muscle growth and regeneration and also undergo myogenic differentiation in dystrophic muscle (Dellavalle et al., 2011). These results suggests that pericytes can occupy the SC niche and form functional myofibres *in vivo*, making them promising candidates in regenerative medicine.
Pericyte population in skeletal muscle is heterogenous and two subpopulations have been identified in mouse muscle: Type-I (Nestin-/NG2+/CD146+/PDGFRα+) and Type-II (Nestin+/NG2+/CD146+/PDGFRα+) (Birbrair et al., 2014, Birbrair et al., 2013c). Both in vitro and in vivo studies revealed that type-II pericytes can differentiate along the myogenic pathway, contributing to muscle regeneration whereas Type-I pericytes can undergo adipogenic differentiation contributing to fat accumulation and/or fibrosis (Au-Nirwane et al., 2017, Birbrair et al., 2013a, Birbrair et al., 2013c, Birbrair et al., 2013b, Gautam et al., 2017).

1.4.2.5 Muscle-derived Stem Cells (MDSCs)

Muscle derived stem cells (MDSCs) are a distinct cell population that can be isolated from primary muscle based on their weak adhesion characteristic using a modified pre-plating method. In this modified pre-plate method, slow adhering cells containing the MDSCs are separated from the fast-adhering cells based on their selective adhesion to collagen coated flasks (Lee et al., 2000, Qu-Petersen et al., 2002, Gharaibeh et al., 2008). These cells were thought to be early myogenic progenitors distinct from SCs and hematopoietic cells but similar to muscle SP cells based on flow cytometry characterisation as CD45-ve/MCadherin-ve/CD34-ve/Flk-1+ve/Sca-1+ve/Desmin+ve cells (Qu-Petersen et al., 2002, Lee et al., 2000).

In addition to their slow adhering characteristics, MDSCs exhibit long -term proliferation capacity both in vitro and in vivo and are multipotent being able to differentiate into myogenic, adipogenic, osteogenic, chondrogenic, and hematopoietic lineages (Qu-Petersen et al., 2002, Torrente et al., 2001, Lee et al., 2000). As well as their myogenic potential in vitro, when transplanted into skeletal muscle and infarcted hearts, MDSCs retained a strong ability to differentiate into skeletal, cardiac, and smooth muscle, integrated into the SC niche and contributed to regenerated myofibres with high regenerative potential (Oshima et al., 2005, Qu-Petersen et al., 2002, Torrente et al., 2001,
Meng et al., 2016). MDSCs have a higher survival rate than SCs and myoblasts due to their resistance to oxidative stress and strong proliferation capacity (Oshima et al., 2005, Qu-Petersen et al., 2002). Furthermore, when compared to myoblasts, MDSCs form dystrophin expressing myofibres more efficiently when directly transplanted into mdx mice (Ikezawa et al., 2003, Qu-Petersen et al., 2002) and displayed superior engraftment in both cardiomyopathic and infarcted hearts of mice following intracardiac implantation (Payne et al., 2007, Oshima et al., 2005). These results suggest that MDSCs could improve regeneration capacity in skeletal muscle and could be of high potential in muscle regeneration.

1.4.2.6 CD133+ cells

Another population of cells actively involved in skeletal muscle regeneration are the CD133+ progenitor cells. Human CD133+ cells are located in the muscle interstitium, under the basal lamina of myofibres and in adult peripheral blood. In addition to their ability to undergo myogenic differentiation in vitro when co-cultured with myogenic cells (Torrente et al., 2004, Benchaouir et al., 2007), human CD133+ cells have demonstrated engraftment potential in dystrophic mice by participating in muscle repair and SC pool replenishment following intramuscular injection (Torrente et al., 2004, Negroni et al., 2009, Meng et al., 2014, Meng et al., 2018).

Importantly in regenerative therapy, both intramuscular and intra-arterial transplantation of genetically corrected human DMD-mutant CD133+ considerably improved muscle shape and function and restored dystrophin expression in immunodeficient mdx mouse muscles (Benchaouir et al., 2007, Torrente et al., 2007).

CD133+ cells have the ability to migrate through blood vessel walls, which is a desirable trait for the development of systemic cell-based therapeutic strategies, making CD133+ cells one of the most promising candidates for DMD cell-based therapy.
1.4.3 Fibro/adipogenic Progenitors in Skeletal Muscle

Apart from myogenic stem/progenitors, other cells such as fibroblasts, endothelial cells, vascular smooth muscle cells, inflammatory resident, as well as infiltrating cells, impact the restoration of skeletal muscle structure (Mierzejewski et al., 2020a). Muscle resident cells found in the interstitium of skeletal muscle that contribute to intramuscular fat formation are called fibro-adipogenic progenitors (FAPs). FAPs are characterised by the expression of platelet-derived growth factor receptor alpha (PDGFRα) and stem cell antigen 1 (Sca1) cell surface marker and can differentiate into adipogenic and fibrogenic but not myogenic lineages. Similar to other muscle-resident cells including muscle stem cells and endothelial cells, they also express CD34 (Uezumi et al., 2010, Dohmen et al., 2022, Joe et al., 2010a, Giordani et al., 2019, Dupas et al., 2011), but not other myogenic markers such as integrin-7 (itga7) and syndecan-4 (Giordani et al., 2019).

Under physiologic conditions, FAPs restore tissue continuity and contribute to muscle repair, and during pathology they contribute to muscle fibrosis and fatty deposition which compromises the tissue environment and potentially limits muscle regeneration indicating a role of FAPs in skeletal muscle pathophysiology. For example, FAPs are required for the maintenance and regeneration of mouse skeletal muscles as FAP depletion resulted in skeletal muscle atrophy, a decrease in the number of SCs, and impaired skeletal muscle regeneration (Wosczyna et al., 2019). In addition, FAPs secrete pro-differentiation factors, IL-6, and IL-10, which play a significant role in the induction of myoblast differentiation (Joe et al., 2010a, Wosczyna et al., 2019, Fiore et al., 2016). On the other hand, during muscle pathology, FAPs proliferate and differentiate resulting in formation of adipose tissue or skeletal muscle ossification (Uezumi et al., 2010, Wosczyna et al., 2012). Moreover, during skeletal muscle regeneration in mdx mice, SC-mediated myotube formation was promoted by FAPs from young mdx mice but suppressed by FAPs from old mdx mice indicating that FAPs play an important role in disease
progression in mdx animals (Mozzetta et al., 2013). Taken together, these findings suggest that FAPs are excellent candidates for therapeutic muscle repair, and their research could pave the way for therapeutic strategies to reduce scarring and fibrosis in chronic disease.

Although MSCs produced from bone and fat, de-differentiated fat (DFAT) cells, embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and, purportedly, SCs are all capable of adipogenic differentiation in vitro, in vivo intramuscular and intermuscular fat is mostly produced by FAPs. The presence of inter and intramuscular fat tissue (also known as marbling) greatly enhances meat sensory quality traits such as flavour, juiciness, texture, and palatability and confer superior sensory properties to meat (Hocquette et al., 2010, Hausman et al., 2014a, Frank et al., 2016). As a result, FAPs have received increased attention in animal science research in the last decade particularly in the field of cultured meat.

1.5 Current Techniques for Isolating and Culturing Muscle Stem/Progenitor Cells

Identification and distinction of the various muscle stem/progenitor cell populations relies on specific cell surface markers which include those belonging to the cluster of differentiation family as well as other surface associated antigens. Selected cell surface markers used for isolation of various muscle stem/progenitor cells are listed on Table 1.1. Some of these markers are unique to a single population, while others are shared with other populations.

Current techniques for isolating muscle stem/progenitor cells include enzymatic dissociation of mononuclear cells from muscle tissue and single fibre or muscle tissue explant culture, each of which is explained in detail below.
Table 1.1 Summary of muscle stem/progenitor cells, surface markers and lineage plasticities described in the literature.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Markers</th>
<th>Lineage potential</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD133+</td>
<td>CD133</td>
<td>Myogenesis \textit{in vivo} and \textit{in vitro}</td>
<td>(Torrente et al., 2007) (Torrente et al., 2004, Benchaour et al., 2007) (Meng et al., 2018, Meng et al., 2014)</td>
</tr>
<tr>
<td>Mesoangioblasts</td>
<td>AP; CD34; TNAP; VE-cadherin</td>
<td>Myogenes</td>
<td>(De Angelis et al., 1999, Bonfanti et al., 2015) (Dellavalle et al., 2007) (Sampaolesi et al., 2003, Sampaolesi et al., 2006) (Diaz-Manera et al., 2010)</td>
</tr>
<tr>
<td>Muscle derived stem cells</td>
<td>CD31, CD34, CD144, C-Kit, Sca1</td>
<td>Myogenesis, Osteogenesis, Angiogenesis, Hematopoiesis</td>
<td>(Gharaibeh et al., 2008) (Huard et al., 2003, Lee et al., 2000, Qu-Petersen et al., 2002)</td>
</tr>
<tr>
<td>Muscle Side population</td>
<td>ABCG2, CD31, CD34, CD45, CD144, MCAM; Sca-1</td>
<td>Hematopoiesis, Angiogenesis myogenesis</td>
<td>(Asakura et al., 2002a) (Meeson et al., 2004) (Lapan et al., 2012a) (Penton et al., 2013) (Tanaka et al., 2009) (Uezumi et al., 2006)</td>
</tr>
<tr>
<td>Myo-endothelial cells</td>
<td>CD34, CD56, CD144, CD146, CD31</td>
<td>Adipogenesis, angiogenesis, and myogenesis</td>
<td>(Tamaki et al., 2002, Zeng et al., 2006)</td>
</tr>
<tr>
<td>Pericytes</td>
<td>CD44, CD73, CD90, CD105, CD146, nestin, NG2, PDGFRβ</td>
<td>Myogenesis, Angiogenesis, Adipogenesis</td>
<td>(Mierzejewski et al., 2020b, Sacchetti et al., 2016, Dvoretskiy et al., 2019, Crisan et al., 2008b, Esteves et al., 2017, Tedesco et al., 2017)</td>
</tr>
<tr>
<td>Satellite cells</td>
<td>CD29, CD34, CD56, CD106, c-Met, M-cadherin, nestin, syndecan 3/4, VCam1</td>
<td>Myogenesis \textit{in vivo} and \textit{in vitro} Adipogenesis Osteogenesis</td>
<td>(Pisani et al., 2010b) (Garcia et al., 2018) (Ding et al., 2017) (Ding et al., 2018) (Liu et al., 2015b) (Stuelsatz et al., 2017) (Maesner et al., 2016) (Laumonier et al., 2017)</td>
</tr>
</tbody>
</table>
### Progenitors lacking myogenic potential

<table>
<thead>
<tr>
<th>Fibro/adipogenic progenitors</th>
<th>CD29, PDGFRα, PDGFRβ, Sca-1, Vimentin</th>
<th>Adipogenesis, chondrogenesis, fibrogenesis, and osteogenesis</th>
<th>(Dohmen et al., 2022, Guan et al., 2017, Joe et al., 2010a, Uezumi et al., 2014a, Uezumi et al., 2010, Fiore et al., 2016, Judson et al., 2017)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblasts</td>
<td>PDGFRα, Tcf4</td>
<td>Fibrosis Extracellular matrix production</td>
<td>(Murphy et al., 2011, Kardon et al., 2003)</td>
</tr>
</tbody>
</table>

Abbreviations: ALP; Alkaline phosphatase; CD; cluster of differentiation; c-kit; cytokine receptor; MCAM, melanoma cell adhesion molecule; NG2; neuroglial 2 proteoglycan; PDGFRα: platelet-derived growth factor receptor α; PDGFRβ: platelet-derived growth factor receptor β; Sca-1, stem cell antigen-1; Tcf4; transcription factor 4; TNAP, tissue non-specific alkaline phosphatase; VCAM-1, vascular cell adhesion molecule-1; VE, vascular endothelial.
1.5.1 Muscle Tissue Dissociation and Enzymatic Digestion

Muscle stem cells reside within the muscle fibres and are often isolated from whole muscle tissue in a multi-step process. First, the muscle tissue is mechanically dissociated into fine fragments using scissors, scalpel or muscle mincer. These muscle fragments are digested using enzymes such as collagenase, dispase, pronase, trypsin in combination, with several digestion rounds resulting in a muscle homogenate containing dissociated single cells, fibre fragments and tissue debris. Subsequently, dissociated single cells are separated from fibre fragments and tissue debris by passing the digested tissues through a cell strainer. Lastly, the cell suspension is centrifuged to remove the supernatant and the isolated cells seeded for proliferation and differentiation. This technique was first developed in rat (Bischoff, 1974) with substantial improvements by Rando and Blau in mouse (Rando and Blau, 1994).

Because muscle tissue contains many different cell types and tissue components, the resulting cells are often mixed populations of myogenic progenitors and non-myogenic cells such as hematopoietic cells, adipocytes, connective tissue fibroblasts and immune cells. As a result, additional purification steps are required to enrich for myogenic progenitors. Currently, there are three main methods commonly used for the purification of muscle stem cells; density gradient centrifugation; pre-plating method and fluorescence activated cell sorting (FACS)/magnetic activated cell sorting (MACS), which take advantage of physical, biological, and molecular features of these cells, respectively.

1.5.1.1 Density Gradient Centrifugation

Following enzymatic digestion, the cells can be separated based on their relative density. The densities of muscle stem cells and other somatic cells differ; therefore, different sub populations can be fractionated by centrifugation with a solution containing dense substrates. First published in a rat study
where 98% of the cells collected between the 50% and 70% interface were myogenic cells (Bischoff, 1997), this method has been used in several species including mouse and chicken with reportedly 43.1% and 70% of the isolated cells being myogenic cells respectively (Yablonka-Reuveni and Nameroff, 1987, Kästner et al., 2000). Several studies have shown the extraction of stem cells from pig muscle using density gradient centrifugation, with varied degrees of purification efficiency (Mau et al., 2008, Ørtenblad et al., 2003, Perruchot et al., 2012, Mesires and Doumit, 2002).

1.5.1.2 Pre-plating Method:

The pre-plating method is based on the varied adhesion properties of distinct cell types. Following enzymatic dissociation, the cell suspension is seeded on uncoated tissue culture plastic. After 1 to 24 hours, fibroblasts and epithelial cells have readily attached onto the culture plate and the myogenic progenitors can be obtained by harvesting the supernatant which can then be seeded unto substrate coated tissue culture dishes (Kästner et al., 2000). In most cases, several rounds of pre-plating are required to enrich for a pure myogenic cell population. This technique has been used to purify myogenic progenitor cells following enzymatic dissociation in several species including mouse (Shahini et al., 2018, Contreras et al., 2018, Gharaibeh et al., 2008, Goetsch et al., 2015), rat (Dai et al., 2015, Dodson et al., 1985), cattle (Dodson et al., 1987, Takahashi et al., 2022) and pigs (Wilschut et al., 2010b, Yang et al., 2017, Li et al., 2015a, Hindi et al., 2017b, Redshaw and Loughna, 2012). However, pre-plating times need to be chosen carefully to maximise cell yield (Syverud et al., 2014, Ding et al., 2017).

1.5.1.3 Fluorescence-activated Cell Sorting (FACS) or Magnetic Activated Cell Sorting (MACS)

Alternatively, prospective isolation of myogenic progenitor cells can be achieved by mechanical and/or enzymatic dissociation followed by fluorescent
activated cell sorting (FACS) or magnetic activated cell sorting (MACS) based on the expression of one or a combination of surface markers. First, muscle is digested with enzymes, and the resulting cell mixture is labelled with specific antibodies that allow myogenic progenitors to be readily identified, before being sorted using a FACS sorter or magnetic separation. FACS relies on physical characteristics of cells, such as forward and side scatter qualities, as well as positive and negative selection by cell surface markers (Liu et al., 2015b). MACS require the cell suspension to be labelled with specific antibodies conjugated to magnetic microbeads (Choi et al., 2021, Syverud et al., 2014). While procedurally MACS is less harmful to cells, FACS allows for more robust cell analysis, more precise cell separation and increased reproducibility thus making it the gold standard for the isolation and study of muscle stem cells (Benedetti et al., 2021, Liu et al., 2015b, Ding et al., 2018).

Although FACS and MACS are frequently used to separate myogenic progenitor cells from a mixed population of mononuclear cells in humans (Webster et al., 1988, Lapan and Gussoni, 2012a, Alexander et al., 2016, Spinazzola and Gussoni, 2017, Pakula et al., 2019, Cerletti et al., 2006, Pavlath and Gussoni, 2005) and rodents (Liu et al., 2015b, Gromova et al., 2015, Feige and Rudnicki, 2020, Maesner et al., 2016, Fu et al., 2015, Stantzou et al., 2017, Hernández-Torres et al., 2020b), there are few reports of the application of this approach in livestock species such as cattle (Ding et al., 2018) and pigs (Ding et al., 2017, Zhu et al., 2022, Choi et al., 2020a) partly due to lack of definitive markers in these species. Another major drawback to utilisation of FACS is that the cell sorting process is stressful for the cells and may potentially alter the gene expression profile and/or cause damage to the cell which may in turn alter their proliferation and differentiation potential.

1.5.2 Single Fibre or Muscle Tissue Explant Culture.

The second main method used to isolate myogenic progenitor cells involves culture of isolated individual myofibres or pieces of muscle tissue (known as explant culture) to allow progenitor cells to migrate out of their niche. This
method was first described in rat and facilitated the study in situ of SCs and their progeny, MPCs, after they have migrated from the tissue (Bischoff, 1986). The explant method was modified to include enzymatic dissociation before culturing the fibres on matrigel coated tissue culture flask which has reportedly led to yield of pure myogenic population as matrigel promoted both SC attachment and proliferation (Conboy et al., 2007, Shefer and Yablonska-Reuveni, 2005). SCs in the basal lamina of cultured fibres have been shown to remain in a quiescent state in low serum media, but migrate out of their niche and proliferate in high serum media (Pasut et al., 2013a). Myogenic progenitor cell migration from skeletal muscle explant onto tissue culture has since been demonstrated to be stimulated by using explant culture in conjunction with matrigel and high serum media in a number of different animals. These cells have been shown to proliferate and maintain their myogenic differentiation capacity in vitro even after prolonged culture (Pasut et al., 2013a, Shahini et al., 2018, Wang et al., 2014, Marg et al., 2020, Hüttner et al., 2019, Stuelsatz et al., 2017, Anderson et al., 2012). Again, only few studies have demonstrated the use of such explant cultures in livestock species including cattle (Ceusters et al., 2012) and pig (Wilschut et al., 2008).

Matrigel and laminin have been shown to maintain proliferation and myogenic differentiation capacity of the primary stem cells in vitro (Wilschut et al., 2010a). Indeed, muscle fibres cultured on uncoated or gelatin coated tissue culture flask gave rise to cells capable of tri-lineage differentiation into adipogenic, chondrogenic, and osteogenic lineages, but not myogenic lineage, indicating that these cells where mesenchymal markers. Thus, matrigel is an important component of the explant culture when the aim is to isolate myogenic progenitors (Ceusters et al., 2017, Wilschut et al., 2008).

Explant cultures are a useful tool for studying SCs and myoblast biology in vitro because of the relative high purity of myogenic cells that can be obtained, however, their use is greatly limited due to their overall poor cell yield.
1.6 Thesis Aims and Objectives

Harnessing muscle stem cells has great potential to study muscle related diseases and improve meat production efficiency, however, the role of muscle progenitor cells in muscle development and regeneration is not well understood, particularly in livestock species. An important technical limitation in those species and specifically in the pig is that stem/precursor cells obtained from muscle are mixed in nature and have low proliferative potential and low differentiation efficiency in-vitro thus limiting their use for research studies or other applications such as cellular agriculture.

The aim of the research presented in this thesis was to develop and characterise an in vitro porcine skeletal muscle model which could be amenable to gene-targeting strategies, such as CRISPR/Cas9, in order to study gene function as it relates to muscle development in pig. The specific objectives were;

1. To isolate, purify, and characterise myogenic cells from porcine skeletal muscle.

2. To develop and characterise an in vitro model of porcine skeletal muscle model using explant cultures.

3. To establish a protocol for CRISPR/Cas9 editing of pig muscle derived progenitor cells.

4. To use the in vitro model developed to study the involvement of the Fibroblast growth factors- β-Klotho (FGF21-KLB) signalling in adipogenic differentiation of pig muscle derived progenitor cells using siRNA and CRISPR/Cas9 loss of function approaches.
Chapter 2 Isolation and Characterisation of Porcine Muscle Progenitor Cells using Fluorescent Activated Cell Sorting.
2.1 Introduction

The pig is an important source of animal protein. In addition, the similarities in organ size and life-span between humans and pigs make the pig a valuable animal model of human physiology and disease (Douglas, 1972, Petersen et al., 2009). Thus, the availability of robust in vitro skeletal muscle models in porcine would aid the development of novel strategies to cure muscular diseases and inform strategies to increase the efficiency of meat production.

A technical limitation to the development of robust in vitro models to study muscle development in the pig or other livestock species is that efficient protocols to isolate and maintain in culture muscle stem/progenitor cells are lacking thus precluding meaningful studies.

Skeletal muscle is a heterogeneous tissue that in addition to muscle fibres contains a variety of specialised cell types such as adipocytes, endothelial cells, fibroblasts, and lymphocytes, as well as different progenitor cell populations including myogenic and non-myogenic (Lapan and Gussoni, 2012b). In addition to satellite cells (SCs) which have been identified as the resident muscle stem cells, a wide variety of progenitor cells with myogenic potential residing in muscle tissue have been described (Péault et al., 2007, Asakura et al., 2002b, Tamaki et al., 2008, Dellavalle et al., 2007, Dellavalle et al., 2011, Mitchell et al., 2010, Pannérec et al., 2013). Studies have suggested that even SC populations in muscle are functionally and molecularly heterogeneous (Motohashi et al., 2014, Rudnicki et al., 2008, Collins et al., 2005, Motohashi and Asakura, 2014, Biressi and Rando, 2010, Chapman et al., 2013). In addition, SCs as well as other muscle progenitor cells act in synergy to contribute to postnatal muscle growth as well as muscle repair and regeneration.

Presently, two main methods are being commonly used for the isolation of SCs and other myogenic progenitors from skeletal muscle and include 1) isolation of mononuclear cells from muscle tissue using enzymatic digestion, and 2) the culture of single fibres / muscle tissue fragments to stimulate the migration of muscle cells (also known as explant culture). While the explant method is
better at preserving the characteristics of the stem cells in their niche, it also results in lower cell yield than the enzymatic approach (Metzger et al., 2020).

The first and more popular approach involves enzymatic digestion and trituration of skeletal muscle to obtain a mononuclear cell population (Hindi et al., 2017b, Danoviz and Yablonka-Reuveni, 2012b). Because skeletal muscle is composed of various type of tissues, cell populations obtained via this method are heterogeneous including fibroblasts, haematopoietic cells and pre adipocytes (Yin et al., 2013, Motohashi and Asakura, 2014). In addition, they contain exceedingly low proportions (typically <5%) of myogenic progenitor cells (Syverud et al., 2014) thus requiring an additional purification step if purer population of myogenic cells are desired. Particularly, in the pig, cells isolated enzymatically from muscle and without further purification show low myogenic potential during in vitro differentiation as evidenced by low fusion index (Choi et al., 2020b, Jeong et al., 2013, Doumit et al., 1993).

The purification of myogenic progenitors from the mixed mononuclear cell pools obtained enzymatically can be achieved using pre-plating method, Percoll density gradient centrifugation, fluorescence-activated cell sorting (FACS) or magnetic activated cell sorting (MACS). Of these methods, Percoll density gradient centrifugation and pre-plating have been more popular approaches reported in pig perhaps because they require no expertise or sophisticated devices (Choi et al., 2020a). Taking advantage of differences in cell density, myogenic cells can be separated from a mixed pool of mononuclear cells when centrifuged through Percoll gradients (Bischoff, 1997), with varying purification efficiencies reported in pigs. In addition, several studies in pigs showed that less than 60% of the cells isolated using this method expressed muscle stem cell marker, neural cell adhesion molecule (NCAM /CD56), as determined by immunostaining, and about 65% were able to form myotubes in vitro (Mau et al., 2008, Ørtenblad et al., 2003, Perruchot et al., 2012, Mesires and Doumit, 2002).

On the other hand, several rounds of pre-plating can be used to separate myogenic cells from muscle extracts taking advantage of the preferential
attachment of fibroblasts to collagen coated surface (Wilschut et al., 2010b, Yang et al., 2017, Li et al., 2015a, Hindi et al., 2017b) although results have also been variable (Syverud et al., 2014).

More robust approaches to purify SCs following enzymatic digestion are fluorescence-activated cell sorting (FACS) and to a lesser extent, magnetic-activated cell sorting (MACS) (Liu et al., 2015b, Spinazzola and Gussoni, 2017, Pakula et al., 2019). The feasibility of these two approaches depends on the availability of suitable antibodies binding appropriate cell surface markers. FACS uses laser-based detection of fluorophore-conjugated antibodies bound to cell surface markers and application of electrical charges to physically separate individual cells, whereas MACS uses magnetic columns and antibody-bound magnetic beads to accomplish this (Hernández-Torres et al., 2020a, Stantzou et al., 2017).

The use of FACS and specific cell surface marker combinations to isolate myogenic progenitors is well established in mice (Liu et al., 2015b, Gromova et al., 2015, Feige and Rudnicki, 2020, Maesner et al., 2016) and humans (Webster et al., 1988, Lapan and Gussoni, 2012a, Alexander et al., 2016, Spinazzola and Gussoni, 2017, Pakula et al., 2019, Cerletti et al., 2006, Pavlath and Gussoni, 2005) but much less in livestock species such as pig (Ding et al., 2017). Some of the surface markers employed alone or in combination in different species include vascular cell adhesion molecule (VCam1), α7-integrin, Syndecan-3/4, C-X-C chemokine receptor 4 (CXCR4), cMet, m-Cadherin, cluster of differentiation 56 (CD56), CD29, CD82 and CD34 (Alexander et al., 2016, Liu et al., 2015b, Maesner et al., 2016, Conboy et al., 2010, Chapman et al., 2013, Gromova et al., 2015, Yin et al., 2013).

CD56 (neural cell adhesion molecule, NCAM) has been reported to specifically mark committed myogenic progenitors in mice (Capkovic et al., 2008), humans (Pisani et al., 2010c) and pigs (Perruchot et al., 2013b). In pig, Ding et al. (2017) utilised FACS to positively select for cells co-expressing CD56 and CD29 and obtained highly purified populations of satellite cells, with 94% of the cells expressing PAX7, and showed that these cells displayed myogenic
potency \textit{in vitro} and \textit{in vivo}. Similarly, Choi et al. (2020a) isolated muscle stem cells from pig muscle using MACS to select cells expressing CD29 and showed that these cells were myogenic \textit{in vitro}. However, in both studies, the adipogenic potential of the cells was not tested. However, Perruchot et al. (2013a) described 2 distinct CD56 cell populations isolated from pig skeletal muscle; while CD56+/CD34− cells displayed only myogenic potency, CD56+/CD34+ cells showed both adipogenic and myogenic potential. In addition, a recent study revealed that CD29+/CD31/54/56- cells isolated from bovine skeletal muscle were fibro-adipogenic progenitor cells (FAPs) distinct from SCs as evidenced by reduced expression of PAX7 and high expression of the FAP marker PDGFRα when compared to SCs (Dohmen et al., 2022). Also, freshly isolated FAPs were shown to differentiate into adipocytes but failed to form myotubes \textit{in vitro} (Dohmen et al., 2022).

CD146, also known as melanoma cell adhesion molecule (MCAM), has been shown to be a surface marker for myogenic progenitor cells in human foetal skeletal muscle (Pujades et al., 2002). Microarray screening of foetal cells revealed that CD146 was strongly downregulated during myoblast differentiation and fusion but it was strongly expressed in proliferating myoblasts \textit{in vitro} (Cerletti et al., 2006). Additionally, when CD146 expression was silenced by RNA interference, myoblasts displayed improved myogenic differentiation and fusion (Cerletti et al., 2006). CD146 has been used alone for enrichment of myogenic cells from human foetal muscle (Lapan and Gussoni, 2012a) and in combination with CD82 to sort myogenic cells from foetal and adult human muscle (Alexander et al., 2016, Persichini et al., 2017). In mice, it has been used to isolate progenitor cells with myogenic potential \textit{in vitro} and \textit{in vivo} (Mierzejewski et al., 2020b). The use of CD146 in purifying myogenic progenitor cells in pig is yet to be reported.

The methods of cell isolation and subsequent purification discussed earlier involve extensive cell manipulation and often result in activation of SCs into a more committed cell type, myoblasts, thus limiting the availability of uncommitted SCs for study (Syverud et al., 2014, Boonen and Post, 2008). In a bid to capture the molecular state of SCs as found in their niche, Machado
et al. (2017) isolated SCs from mice muscle tissue fixed in situ with 0.5% paraformaldehyde (PFA) (T0-SCs) and compared their transcriptomes to those of SCs isolated using a standard isolation protocol from unfixed tissue (T3-SCs). Their results showed that the standard protocol induced significant transcriptional changes in the cells which involves induction of a large number of genes (Machado et al., 2017). In addition, the T3-SCs also displayed a transcriptional profile indicative of a switch from quiescence to activation and proliferation, with upregulation of genes implicated in early response and dissociation-induced genes. On the contrary, the quiescence-enriched genes related to fatty-acid metabolism were downregulated in the T3-SCs (Machado et al., 2017) suggesting a metabolic switch from fatty-acid oxidation to glycolysis during muscle stem activation (Ryall et al., 2015). Thus, the overall consequences of isolation procedures on the transcriptional signatures of targets cells both need to be considered and require further investigation.

2.2 Preliminary Data

In order to set up FAC sorting of myogenic progenitor cells, the specificity of cell surface marker antibodies for muscle SCs in pig was tested. Cryosections of pig skeletal muscle were stained with antibodies against laminin, PAX7, CD146 and CD56. CD146 was localised around the basement laminar of the muscle fibre as indicated by laminin immunofluorescent staining (Figure 2.1A). Both CD146 and CD56 co-localised with PAX7 in porcine muscle sections (Figure 2.1B, Figure 2.2) indicating that they are suitable surface markers for isolation of porcine myogenic cells (Rebecca McCall, unpublished data).
Figure 2.1 Immunofluorescent staining to identify potential surface markers for myogenic cells in sections of porcine muscle tissue.

(A) Double staining for laminin (red) and CD146 (green) in sections of muscle tissue in pig. (B) Immunostaining for nuclear PAX 7+ (red) and cell surface marker CD146 (green) showing co-localisation of CD146 surface marker (green) with satellite cell specific marker PAX 7 (red) (white arrows in insert). Nuclei in all images shown was stained with 4′,6-Diamidino-2-phenylindole (DAPI; blue). Scale bars = 100µm and 10µm and in A and B respectively.
Figure 2.2 Immunofluorescent staining to identify potential surface markers for myogenic cells in sections of porcine muscle tissue.

Immunofluorescent staining for CD56 (green) and PAX7 (red) on pig muscle sections with arrowhead indicating satellite cells expressing both markers. Nuclei in all images shown was stained with 4',6-Diamidino-2-phenylindole (DAPI; blue). Scale bars = 25µm.
2.3 Objective

The main objective of the work described in this chapter was to develop FACS procedure for isolating myogenic and adipogenic precursor cells from pig skeletal muscle and to characterise those cells.

2.4 Experimental Aims

The specific aims include:

1. To establish a FACS protocol using the cell surface marker, CD146, for isolation of myogenic progenitor cells from porcine skeletal muscle.
   
   a. To compare the expression of lineage specific markers between the CD146+ and CD146- cell fractions.
   
   b. To determine the relative adipogenic and myogenic differentiation potential of the CD146+ and CD146- cell fractions.

2. To establish the feasibility of using FACS and PFA fixation for isolating myogenic progenitor cells that could be suitable for subsequent transcriptome analyses.
2.5 Materials and Methods

2.5.1 Animals and Tissue Collection

This study was carried out with approval from The Roslin Institute’s (University of Edinburgh) Animal Welfare and Ethical Review Board and in accordance with the UK Animals (Scientific Procedures) Act, 1986.

Male piglets (1 week old) from three Large White X Landrace litters kept at Howgate farm (Edinburgh) were euthanized by intravenous injection of sodium pentobarbitone 20% w/v (Henry Schein Animal Health, Dumfries, UK) and subsequent decapitation. Upon confirmation of death, the skin and connective tissues of the hind limb were removed to expose the musculature. The Semitendinosus (ST) muscle was identified (Figure 2.3) and dissected aseptically. Muscle samples (1-2 g) were either transported on dry ice for later RNA/immunohistochemistry analysis or placed in phosphate buffered saline (PBS) supplemented with 2.5µg/ml Amphotericin B (Gibco, Thermo Fisher Scientific cat #15290-026), and 1% penicillin-streptomycin (PS) (Gibco, Thermo Fisher Scientific cat #1540-122) (PBS+F+PS solution) and transported on ice for cell isolation and culturing.

In the lab, samples for immunohistochemistry (IHC) were embedded in Optimum Cutting Temperature compound (OCT) on a pre labelled mould which was subsequently dipped into isopentane chilled by placing on dry ice. The isopentane treatment was done for 20 seconds after which the sample on mould was stored at -80°C until sectioning. Samples for RNA analysis were directly stored at -80°C until required.
Figure 2.3 Pig hindlimb showing the hamstring muscles; Biceps femoris muscle, semitendinosus muscle and semimembranosus muscle.

Adapted from Mini Pig info, available online at https://www.minipiginfo.com/pig-anatomy-and-terminology.html
2.5.2 Isolation of Progenitor Cells From Fresh Muscle Tissue

In the laboratory, precursor cells were isolated from the muscle tissue by mechanical dissociation and enzymatic digestion following a protocol adapted from (Lapan and Gussoni, 2012). Briefly, the muscle was further cleaned of fat and connective tissue and around 10 g of tissue from the middle portion was weighed out and transferred to a falcon tube. This tissue was washed in 3 changes of cold PBS+F+PS solution. Subsequently, muscle tissues were minced into a fine paste with sterile scissors and scalpels on a petri-dish placed on ice and digested for 1 hour in digestion medium composed of Dulbecco’s modified Eagle’s medium (DMEM) high glucose, (Life Technologies, cat #41966-029) supplemented with 2mg/ml type II Collagenase (Sigma Aldrich, cat # C6885-100MG), 3.5% Bovine Serum Albumin (BSA, Cytiva HyClone™, cat# SH30574.02) and 40 µg/ml DNAse (Sigma Aldrich, cat# D5025-15KU) at 37°C under agitation (70 rpm) to allow enzymatic digestion of muscle fibres and dissociation of the cells. Digestion media was added at 10 ml per gram of tissue. The digesting tissue was triturated with 25 ml strippette (10x), followed by 10 ml strippette (10x) and finally 5 ml strippette (10x) every 20 min to aid further release of cells from the tissue. After 1 hour, enzyme activity was neutralised by addition of equal volume of fresh DMEM supplemented with 20% Foetal Bovine Serum (FBS, Life Technologies, Thermo Fisher Scientific, cat #10500-064) and 1% PS. The resulting muscle extracts were sequentially filtered through 100 µm, 70 µm and 40 µm cell strainer (Fisherbrand™, Fisher Scientific, cat# 22-363) and centrifuged at 2000 rpm for 10 minutes (min) to pellet the cells. The supernatant was discarded, and pellet resuspended in known volume of media supplemented with 10% FBS. The number of cells obtained was counted with a Neubauer chamber for each animal using trypan blue dye exclusion. The cells obtained after the digestion were expanded in culture for later experiments or stained for FACS sorting.
2.5.3 *In Situ* Fixation and Isolation of Skeletal Muscle Stem Cells

Mononuclear cells from three 4-weeks old male piglets were extracted from muscle tissue fixed in paraformaldehyde (PFA) *in situ* following a protocol adapted from (Machado et al., 2017). Briefly, 10 g of muscle tissue was dissected from the middle portion of the semitendinosus muscle, rapidly chopped into smaller pieces, and immediately transferred into a falcon tube containing ice cold PFA (0.5%). The pieces of muscle tissue were further minced into a fine paste and incubated in PFA (0.5%) for 1 hour with gentle rotation at 4°C. After 1 hour, the fixed muscle tissue was washed 3 times in cold PBS+F+PS solution to remove residual PFA. Then, the fixed tissue was incubated for 90 min in digestion media containing DMEM supplemented with 4mg/ml type II Collagenase, 3.5% BSA, 80µg/ml DNAse and 2 mg/ml Dispase (Sigma Aldrich, cat # D4693) at 37°C with agitation at 70 rpm. Subsequently, muscle extracts were filtered, and cells counted as described in section 2.5.2 above. Cells were either used for RNA extraction or stained for FACS/ Flow cytometry analysis. Of note, to compare between cells from fixed and live tissue, mononuclear cells were simultaneously extracted from fresh muscle for each animal using the protocol described in section 2.5.2.

2.5.4 Flow Cytometry Analysis and Fluorescence-Activated Cell Sorting (FACS)

In order to isolate myogenic and non-myogenic cell populations, muscle cell extracts (10^7 cells per animal) were prepared for FACS as follows. First, non-specific binding was blocked by incubation in 10% mouse serum (Sigma Aldrich, cat # M5905-10ML) in PBS at 4°C for 30 min and cells subsequently stained for 1 hour at 4°C with antibodies against CD146-AF647 (AbD Serotec-BioRad, cat# MCA2141A647), CD45-FITC (BioRad, cat# MCA832F) and CD31-RPE (AbD Serotec-BioRad, cat# MCA1746PET). Unstained control and Isotypes including mouse IgG1-AF647 (AbD serotec-BioRad, cat# MCA928A647), mouse IgG1- FITC (AbD serotec-BioRad, cat# MCA928F), and mouse IgG1-RPE (AbD Serotec-BioRad, cat# MCA928PE) were used as
negative controls. In addition, Fluorescence minus one (FMO), comprising all antibodies except CD146-AF647 was used as another control to set up the gating for FACS. To determine cell viability, DAPI was added to the samples immediately prior to FACS. Finally, the cells were sorted on a BD FACSArray Fusion (BD Biosciences, San Jose, CA, USA).

For comparing fixed versus non-fixed tissues (Figure 2.11 and 2.12), mononuclear cells were prepared for FACS using the staining strategy described above except that CD56-BV421 (BD Biosciences, cat# 550760) and the corresponding isotype control, mouse IgG1-BV 421 (BD biosciences, cat# 562748) was used instead of CD146 antibody. In addition, cell viability was determined by the addition of 5 µM SYTOX™ Orange Nucleic Acid Stain (Invitrogen, cat# S11368) instead of DAPI.

Prior to cell sorting by FACS, to ensure positive binding of the individual antibodies to the target cells, muscle extract cells from fixed and fresh tissue were stained as described for FACS with primary antibodies including CD34, CD56, and CD146 and their corresponding isotype control. In addition, 1µM SYTOX™ Blue Dead Cell Stain (Invitrogen, cat# S34857) was added to the live cells only prior to flow analysis to determine cell viability. Flow analysis was performed on BD Fortessa X20, and data analysed with FACSDiva software (BD Biosciences, San Jose, CA, USA).

### 2.5.5 Myogenic Differentiation

CD146+ and CD146- cells were seeded on rh-Laminin 521 (0.5 µg/cm²; Gibco-Life Technologies, cat# A29248) coated 24-well plates at a density of 5 ×10⁴ cells per well (in triplicate) in growth media (DMEM supplemented with 10% FBS; 5ng/ml bFGF and 1% PS) and incubated at 39°C until they were 70% confluent. At this point, the media was changed to muscle proliferation media (80 nM dexamethasone; Sigma Aldrich; cat# D4902; 10% FBS and 1% PS) until 100% confluence. Myotube formation was induced by incubation in serum free medium supplemented with 1% Insulin-Transferrin-Selenium (100X, Gibco-Thermofisher scientific, cat# 41400045) and 1% PS at 39°C for 5 days with media being changed twice. Samples were taken at days 0, 3 and
5 in TRIzol reagent and stored at -80°C for gene expression analysis while some wells were fixed in 4% PFA for immunocytochemistry on day 5.

2.5.6 Adipogenic Differentiation

For adipogenic differentiation, CD146+ and CD146- cells were seeded onto 24-well plate coated with collagen (50 µg/ml, Sigma Aldrich, cat# C7661-10 MG) at a density of 5 x 10^4 per well (triplicate wells) in growth media (DMEM HG, 10% FBS, 5 g/ml bFGF, 1% PS) until 90% confluence. Cells were induced to differentiate in the presence of 3-isobutyl-1-methylxanthine –IBMX (0.5 mM, Stemcell Technologies, cat# 72762), dexamethasone (1 µM, Sigma Aldrich cat #D4902), indomethacin (100 µM, Stemcell technologies cat#73942), Insulin (10 µg/ml, Sigma Aldrich, cat#19278), 10% FBS and 1% PS. After 4 days, the media was replaced with adipocyte maintenance media (DMEM supplemented with 10% FBS, 10 µg/ml Insulin and 1%PS) until day 14 with media changes every 2-3 days. Samples were taken at days 0 and 7 and were harvested in TRIzol for RNA extraction and gene expression analysis.

2.5.7 RNA Extraction from Fresh Cells

RNA isolation from cells was performed using Trizol reagent following the manufacturer’s instructions. All centrifugation was performed at 12,000 x g (11,400 rpm) in a table-top centrifuge unless otherwise stated.

The wells with cells were washed once in PBS followed by addition of cold TRIzol (500 µl or 1 ml per well in 24 well plates or 12 well plates respectively or 500 µl per 1x10^6 cells if cell number was determined prior to extraction). For the cells in wells, the plates were gently agitated to dislodge the cells before transferring to clean nuclease free 1.5 ml Eppendorf tubes and stored at -80°C until required for extraction. To isolate RNA, the samples were thawed on Ice, vortexed briefly for 10 seconds to homogenise the sample and incubated at room temperature for 5 min. Then 100 µl (for 500 µl Trizol) or 200 µl (per 1 ml of TRizol) of Chroloform (ThermoFisher Scientific UK, cat# 67-66-
3) was added to the sample, vortexed for 10 seconds (secs) and then incubated for 3 min at room temperature. The samples were then centrifuged for 15 min at 4°C to obtain 3 phases (a DNA containing lower red phenol chloroform phase, an opaque interphase containing proteins and upper colourless aqueous phase containing RNA).

The aqueous phase was carefully transferred to a clean nuclease free 1.5 ml Eppendorf tube and 250 µl or 500 µl of propan-2-ol (Fisher Scientific, cat # 67-63-0) to 500 µl or 1 ml of Trizol reagent respectively was added to facilitate RNA precipitation. For small number of cells, to facilitate visualisation of RNA pellet, RNA grade glycogen (5 µg, ThermoFisher Scientific, cat# R0551) was added to the mixture and then samples were incubated at room temperature for 10 min followed by centrifugation for 10 min at 4°C to pellet the precipitated RNA. The supernatant was discarded, and the RNA pellet was washed with 500 µl or 1 ml of 75% ethanol (to 500 µl or 1ml of Trizol reagent respectively) and centrifuged at for 5 min at 7500 x g and 4°C. The supernatant was discarded and the pellet air dried for 10 to 20 min. When dry, the RNA pellet was resuspended in 15 µl of nuclease free water.

The RNA concentration was quantified using a Nanodrop spectrophotometer (NanoDropTM 1000, Thermo Scientific) and stored at -80°C until required.

2.5.8 RNA Extraction from Formalin Fixed Cells

Following thawing, 1.0*10^6 cells were expanded in culture for 2 days, harvested with trypsin and divided into 3 groups containing 1.0*10^6 cells each. The cells were incubated for 30 min on ice with 0.5% and 2% PFA for group A and Group B respectively while Group C was not fixed. After cells were washed 3 times in PBS. RNA from fixed cells as well as mononuclear cells isolated from in situ fixed muscle tissue was extraction using the RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion, cat# AM1975) following the protocol described in (Machado et al., 2017). Briefly, cells were digested with protease for 1 hour in digestion buffer at 50°C. Once digestion was
completed, nucleic acid was isolated by addition of isolative additive, transferred to the filter cartridge, and centrifuged at 10,000 x g for 30 secs at room temperature to pass the mixture through the filter. After a couple of washes, the bound RNA was treated with DNase mix and incubated for 30 min at room temperature to purify the RNA. Lastly, the purified RNA was washed a couple of times followed by addition of elution solution which was allowed to sit for 1 min and subsequently centrifuged at 10,000 x g for 1 min to elute the RNA. The RNA was quantified spectrophotometrically using a Nanodrop ND-1000 (Labtech International Ltd) and the quality assessed electrophoretically using a Tapestation 2200 (Agilent Technologies). Note that to ensure comparability in these experiments, RNA from both the live and fixed cells were isolated using RecoverAll™ Total Nucleic Acid Isolation Kit with the incubation step at 50°C performed for only 15 min for live cells.

2.5.9 Complementary DNA (cDNA) Synthesis and Gene Expression Analysis

The RNA was reverse transcribed in a 2-step reaction using the SuperScript III RT kit (Invitrogen, cat #18080-044) according to the manufacturer's instructions. RNA samples were diluted with water to a concentration of 0.5 µg/13 µl and mixed with 1 µl of Random Primers (Promega Cat #C-1181) (which contain random sequences and is able to bind to its complementary sequences on the RNA template thus serving as a starting point for synthesis of a new strand) and 0.5 µl of dNTP mix (Invitrogen cat #18427-013) to help with DNA amplification. The mixture was heated to 65°C in a thermocycler (Biometra, USA) for 5 min to anneal the primers and subsequently cooled to 4°C for 5 min. Then a master mix containing 1 µl of RNasin Plus Rnase Inhibitor (Promega cat #N2611), 4 µl of 5X First-Strand Buffer, 1 µl 0.1DTT, and 1 µl SuperScript III (all components of superscript III kit) was added and mixed by pipetting. While Rnasin plus inhibitor prevents RNA degradation, DTT helps with the optimal activity of the reverse transcriptase. The mixture is heated to 25°C for 5 min (to allow for extension of the primers), 50°C for 1 hour (for DNA
polymerisation/cDNA synthesis) and at 70°C for 15 min (to inactivate the enzyme) in a thermocycler. The cDNA samples were used immediately or stored at -20°C.

For gene expression studies, quantitative real time polymerase chain reaction (qRT-PCR) was performed using Sensi-FAST SYBR Lo-ROX Kit (Bioline UK, cat #BIO-94050) according to manufacturer’s instructions on the Stratagene Mx3005P machine (Agilent, La Jolla, CA. USA). Briefly, 2 µl of diluted cDNA (8-fold dilution) was mixed with 5 µl SensiFAST SYBR Lo-ROX Kit, 0.4 µl forward primer (100 µM), 0.4 µl reverse primer (100 µM) and 2.2 µl nuclease-free water per 10 µl reaction. cDNA was amplified in a 3-cycle protocol involving initial denaturation and polymerase activation at 95°C for 2 min, followed by 40 cycles of 5 secs at 95°C (denaturation), 11 secs at 60°C (DNA annealing) and 5 secs at 72°C (DNA extension) followed by a final extension cycle of 1 min at 95°C. Finally, samples were heated for 30 secs at 60°C and 30 secs at 95°C to generate the melt curve and measure fluorescence. Included with each analysis was No reverse transcriptase (RT) control, No DNA template control and a standard curve generated from 4-fold serially diluted samples obtained from a pool of all the samples being analysed. The standard curve was used to generate the quantity copies (arbitrary units) of the gene in the samples. All samples and controls were run in duplicates and the results generated analysed on the MxPro software and excel spreadsheet. All samples were normalized against two reference genes (18s and RPL4). The primers sequences used for gene analysis are listed on Table 2.1.
<table>
<thead>
<tr>
<th>Primer SYMBOL</th>
<th>Primer Name</th>
<th>Forward Primer Sequence 5’-3’</th>
<th>Reverse Primer Sequence 5’-3’</th>
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<tr>
<td>18s</td>
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<td>GCTGGCACCAGACTTG</td>
<td>GGGGAATCAGGGGTTCG</td>
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<td>CD31</td>
<td>Cluster of differentiation 31</td>
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<td>Myogenic factor 5</td>
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</tr>
<tr>
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<td>Description</td>
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</tr>
<tr>
<td>--------</td>
<td>--------------------------------------</td>
<td>------------</td>
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2.5.10 Immunofluorescence Analysis

For immunocytochemical staining, cells were pre-fixed for 2 min by adding equal volume of pre-warmed 4% PFA to the culture media to prevent cells lifting during washing. After washing 2 times for 5 min each in PBS, the cells were fixed in 4% PFA for 15 min at room temperature followed by three washes in PBS. Next, the fixed cells were permeabilised by incubating them for 10 min in 1:1 methanol: acetone solution at room temperature. Next, the samples were treated with protein block solution (abcam, cat# ab64226) to prevent nonspecific antibody binding. Subsequently, the cells were incubated overnight at 4°C with the primary antibody (anti-myosin heavy chain (MYHC) 5 μg/ml, R&D laboratories, Huntingdonshire, UK; cat#NMAB4470) diluted in antibody diluent reagent (ThermoFisher Scientific, Uk; cat# 003118). The next day, after washing the cell in PBS (3 times, 5 min each) to remove any unbound primary antibody, the cells were incubated for 1 hour with secondary AF488-conjugated goat anti-mouse antibody (Invitrogen, cat# A11029) at room temperature.

Next, the cells were washed in PBS for a further 3 times (5 min each) and overlayed with a mountant containing 4′,6-Diamidino-2-phenylindole (DAPI) (Fluoroshield with DAPI; Sigma, cat #F6057) to counterstain the nuclei and subsequently covered with a coverslip. Images were captured on an Axiovert 25 and analysed on the Zen Software (Zeiss, Oberkochen, Germany).

2.5.11 Oil Red 0 Staining

Adipocytes were visualised by staining with Oil Red O (ORO; Sigma-Aldrich, cat# 00625). Briefly, Cells were fixed in 4% PFA for 15 min, washed in PBS (2times, 5min each) and incubated with 0.4% ORO (diluted in isopropanol and filtered) for 10min at room temperature. Subsequently, cells were washed in distilled water (5times, 5min each) and left in PBS until imaging. Images were captured with a Zeiss Axiovert 25 inverted microscope.
2.5.12 Statistical Analysis

All Graphs were produced using GraphPad PRISM software (version 9.0.2; La Jolla, CA USA) with data presented as mean ± SEM. Statistical analyses were performed using Minitab 20 Statistical Software (2022) (Computer software, Pennsylvania, USA). Data were assessed for normal distribution using the Kolmogorov–Smirnoff test (P>0.01) and log-transformed before further analyses if not normally distributed. Unless otherwise stated, statistical significance was determined using one-way or two-way ANOVA and a subsequent comparison among means with Tukey’s test with statistical significance set at P<0.05.
2.6 Results

2.6.1 Isolation of myogenic progenitor cells by FACS based on the cell surface marker, CD146.

2.6.1.1 FACS of CD146+ and CD146- cell populations from porcine skeletal muscle mononuclear cells.

CD146 had been reportedly used successfully to sort human myogenic cells (Lapan et al., 2012a, Lapan and Gussoni, 2012b). Thus, the finding that CD146 co-localises with PAX7 in pig skeletal muscle (Figure 2.1), suggests that it could be used as a surface marker to isolate myogenic cells in this species. Therefore, I sought to isolate myogenic progenitor cells from pig skeletal muscle extracts using fluorescence activated cell sorting (FACS) with antibodies targeting CD146.

Figure 2.4 shows representative scatterplots of the FACS procedure used to prospectively isolate myogenic and non-myogenic fractions from muscle extracts, namely CD45−/CD31−/CD146+ and CD45−/CD31−/CD146- fractions, respectively. These fractions were sorted sequentially and events were visualised in a side scatter area vs forward scatter area plot (SSC-A vs FSC-A, Figure 2.4A). Then, in a forward scatter height vs forward scatter area plot (FSC-H vs FSC-A, Figure 2.4B) single cells were selected, and cellular debris excluded. Next DAPI was used to select live cells (DAPI- negative; Figure 2.4C) followed by exclusion of CD45-FITC-positive haematopoietic cells (Figure 2.4D, 25.7 ± 6.4% of total). The number of live cells obtained was comparable between the stained samples and the unstained control (39.3 ± 4.9% vs 38.8±6.6% of total). Finally, in a double plot of CD146-AF647 versus CD31-RPE, the CD45 negative cells were sorted into CD146 positive/CD31 negative (CD146+/CD31-) cells and CD146 negative/CD31 negative (CD146-/CD31-) cells (Figure, 2.4E). Thus, fractions obtained separately were CD45-/CD31-/CD146+ (CD146+ cells, 2.4± 0.7 % of total) and CD45-/CD31-/ CD146- (CD146- cells; 17.80 ± 7.3 % of total), prospectively corresponding to myogenic and non-myogenic populations respectively. Unstained cells (Figure 2.4f and 2.4g), antibody isotypes (Figure 2.4h and 2.4i), or fluorescent minus one (FMO) (Figure 2.4j), were used as controls. The CD146+ and CD146- cells collected were then cultured and characterized.
Figure 2.4 FACS Isolation of CD146+ and CD146- cells from mononuclear cells of porcine skeletal muscle tissue.

(A) Selection of single cells plotted as FSC-H vs FSC-A based on cell size. (B) Dot-plots showing exclusion of cell fragments or non-cellular material from single cells obtained in (A) based on cellular granularity/complexity in an SSC-A vs FSC-A plot. (C-E) Plots showing series of events to select DAPI-negative (C) and CD45(FITC)-negative cells (D) to obtain live, hematopoietic-negative cells. (E) Plots for CD146+/31- and CD146-/31- cells collected. (f-j) Controls showing unstained cells for CD45 (f), CD146 and CD31 (g). (h-i) Isotype controls for CD45 antibody conjugated to FITC (h), CD146 antibody conjugated to AF647 and CD31 antibody conjugated to RPE (i). (j) Fluorescent minus one control for CD146 (AF647) antibody.

FSC-A forward scatter area; FSC-H forward scatter height; SSC-A side scatter area; SSC-H side scatter height.
2.6.1.2 Expression of Cell Surface and Lineage-specific Markers by Sorted Cell Fractions

The expression of selected surface markers as well as lineage-specific transcription factors was analysed in CD146+ and CD146- cells by RT-qPCR. Results indicated that, as expected, CD146+ cells expressed significantly higher levels of CD146 mRNA compared to the CD146- fraction (P<0.001; Figure 2.5A). Moreover, levels of PAX7 and CD56, both of which are selectively associated with myogenic progenitors, were also distinctly higher in CD146+ cells (P<0.001, Figure 2.5 B-C). In contrast, expression of PDGFRα, a cell surface marker of adipogenic precursors, was significantly lower in the CD146+ fractions compared to the CD146- fraction (P<0.05, Figure 2.5D), whereas levels of the pre-adipocyte-specific transcription factor, ZNF423 and the cell surface marker CD34, tended to be lower in the CD146+ fractions compared to the CD146- fractions (P=0.068; Figure 2.5E and P=0.079; Figure 2.5F) for CD34 and ZNF423 respectively.
Figure 2.5 Gene expression levels of muscle and adipose cell markers in sorted cell populations (CD146+ and CD146-).

Relative mRNA levels of myogenic cell markers, CD146 (A), PAX 7 (B), and CD56 (C); adipose cell markers PDGFRα (D) and ZNF423 (E); and cell surface marker CD34 (F) as measured by reverse transcriptase quantitative real-time PCR in CD146+ (gray bar) and CD146- (white bar) cells. 18s was used as housekeeping gene, n = 6; bars are mean ± SEM. P values shown for each graph.
2.6.1.3 In vitro Differentiation Potential of CD146+ and CD146- Fractions

The ability of CD146+ and CD146- cell populations to undergo differentiation into myogenic or adipogenic lineages was tested in vitro. After 3 days in myogenic conditions, CD146+ cells were able to fuse and form myotubes while CD146- cells failed to form any myotubes as shown by MYHC staining (Figure 2.6A). In control media, both CD146+ and CD146- cells did not form myotubes (Figure 2.6A, left, bottom and top panel). This was confirmed by the increase in the level of expression of the myogenic markers MYHC3 and MYOG in CD146+ compared to CD146- fractions (P<0.01; Figure 2.6B and P<0.05; Figure 2.6C). Unstained cells, IGG negative control and a secondary control (antibody only) were used as controls. On the contrary, when cultured in adipogenic media, both CD146+ and CD146- cells differentiated into multilocular adipocytes as shown by oil red O staining (Figure 2.7A). However, the level of differentiation was higher in CD146- compared to CD146+ cells as indicated by the expression of the lipid transport protein (FABP4) (P<0.05; Figure 2.7B).
Figure 2.6 Myogenic differentiation of CD146+ and CD146- cells.

(A) Representative immunofluorescence images of undifferentiated (left) and differentiated (right) cells from CD146+ (top) and CD146- (bottom). Cells fused to form multinucleated myotubes after 3 days under myogenic conditions in CD146+ fractions (top right) but not CD146- fraction (bottom right). Scale bar= 100 µm. (B) Quantification of muscle marker (MYHC3, MYOG) expression by RT qPCR in undifferentiated (Day 0) and differentiated (Day 3 and Day 5) CD146+ and CD146- cell samples. Results shown as Mean ± SEM, P values shown on graphs.
Figure 2.7. Adipogenic differentiation of CD146+ and CD146- cells.

(A) Representative oil red O-stained images of differentiated adipocytes from CD146+ and CD146- cell fractions. Scale bar= 100μm. (B) mRNA levels measured by RT qPCR of FABP4 before and during adipogenic differentiation of CD146+ and CD146- cells. Results shown as Mean ± SEM, P values shown on graphs.
2.6.2 FACS Analyses of In Situ Fixed Tissues

2.6.2.1 mRNA Yield and Quality following Fixation of Cells With PFA

One objective of my study was to compare the transcriptional profile of quiescent and activated pig SCs. To obtain quiescent SCs, I followed a published protocol (Machado et al., 2017) involving in situ fixation of the muscle tissue before cell extraction, i.e. to prevent SCs from becoming activated during extraction. As a preliminary step, I tested the effect of fixation of cells with PFA on the quality and yield of mRNA. For this experiment, mononuclear cell extracts from pig muscle tissue were used. Subsequently, cells that were either fresh or fixed in 2% PFA or 0.5% PFA were lysed, and RNA was extracted. The results showed comparable RNA integrity numbers (RIN) between fixed and non-fixed cells, as demonstrated by the presence of intact 28S and 18S bands (Figure 2.8A). Similarly, both fixed and non-fixed cells had comparable RNA yields (Figure 2.8B). More so, increasing the concentration of PFA did not have any negative effect on RNA yield or quality. Taken together, the result indicates that fixation with PFA did not alter RNA quality or yield.

![Figure 2.8](image)

Figure 2.8 Assessment of RNA yield and integrity.

(A) RNA was isolated from pig primary cells following fixation with either 0.5% PFA or 2% PFA or from live cells without any fixative and analysed on the Agilent Tapestation 2200. The gel image shows the separation profile of each sample along with the RIN. All tested cell types showed visible 28S and 18S subunits at 4000 bp and 2000 bp respectively. (B) Yield of total RNA recovered from each cell type as measured on the nanodrop spectrophotometer.
2.6.2.2 FACS of Fixed vs Fresh Muscle Cells using CD56 Antibody.

For this experiment, I sought to use FACS based on the expression of CD56 as an alternative approach for purifying myogenic cells, given that like CD146, CD56 co-localises with PAX7 in pig skeletal muscle (Figure 2.2), and moreover, CD56 had been reportedly used successfully to sort pig myogenic cells (Ding et al., 2017). Flow cytometry analysis showed that both CD56 and CD146 gave a distinct peak of positively stained cells compared to their respective isotype controls and unstained cells (Figure 2.9A). Moreover, most of the CD146+ cells were also positive for CD56 (Figure 2.9B) indicating that CD56 can be used to purify pig myogenic cells.

To determine the suitability of in situ fixed cells for subsequent FACS isolation of myogenic precursor cells, I compared the antibody binding profile of candidate antibodies between fixed and fresh cells. To do this, fresh or fixed cells were extracted as described in section 2.5.2 and 2.5.3 respectively, and were stained with antibodies against CD34, CD56 and CD146 prior to analyses by flow cytometry. Doing this, similar profiles were obtained between live and fixed cells for all antibodies (Figure 2.10A and 2.10B). Particularly, the light scattering patterns were similar between live and fixed cells as shown on the SSC-A versus FSC-A dot plots in Figure 2.10A and Figure 2.10B respectively. Again, my results indicate that fixation of pig muscle tissue before cell isolation does not alter antibody binding.
Figure 2.9 Analysis of cells using flow cytometry and CD56 and CD146 antibodies.

(A) Histograms of stained cells showing majority of cells stained positively with CD56-BV421 and CD146-AF647 antibodies (orange peak on the right). Peak on the left represents signal from unstained (red) and isotype-controls (blue). (B) CD146-AF647 and CD56-BV421 double plot showing CD146+/CD56+ (double positive cells (Q14)).
Figure 2.10 Flow cytometry profile of fixed and live cells stained with selected antibodies and their respective isotype control.

(A) Representative flow cytometry profile of cells extracted from fresh tissue displaying events as dotplots and showing the profile of cells stained with isotype controls for CD34, CD56 and CD146 antibodies (top lane) compared to displacement of cells stained with CD34, CD56 and CD146 antibodies respectively (bottom lane). (B) Representative flow cytometry profile of cells isolated from muscle tissue fixed with 0.5% PFA displaying events as dotplots and showing the profile of cells stained with isotype controls for CD34, CD56 and CD146 antibodies (top lane) compared to displacement of cells stained with CD34, CD56 and CD146 antibodies respectively (bottom lane). Each representation is illustrated by the isotype control (top associated to its corresponding positive staining (bottom). SSC- side scatter, FSC- forward scatter.
Having established the compatibility of \textit{in situ} fixation and FACS antibody binding, I proceeded to prospectively sort myogenic (CD56+) and non-myogenic progenitors (CD56-) from the live and fixed cells using FACS. FACS gating for live and fixed cells is shown in Figure 2.11. Based on cell size, single cells were selected in FSC-H vs FSC-A dot plots (P2 in Figure Ai and Bi). Then Sytox orange negative live cells were gated in the fresh cells only. (Figure 2.11 Aii). Sytox orange was not added to the fixed cells as they will all take up Sytox orange, the live gate in Bii was only applied to the fixed cells to visualise the cells and was removed for subsequent analysis. The live cells from fresh muscle tissue (in Aii) and all single cells gated in P2 from fixed tissue (Figure Bi) were further analysed to exclude CD31-FITC postive and CD45-FITC postive endothelial and haematopoietic cells, respectively (Figure 2.11 Aiii and Biii). Lastly, CD31-/CD45- (FITC) cells were sorted into CD56-BV421-positive (CD56+) and CD56-BV421-negative (CD56-) fractions (Figure 2.11 Aiv and Biv).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{facs_isolation.png}
\caption{FACS Isolation of CD56+ and CD56- cells from fresh or in situ fixed mononuclear cells.}

(Ai, Bi) Dot plots of events in FSC-H vs FSC-A plot to select single cells and exclude non cellular debris. (Aii) Selection of Sytox orange-negative live cells and (Bii) visualisation of fixed cells on sytox orange PE vs FSC-H plot. Selection of CD45-/CD31-(FITC) cells to obtain live haematopoietic negative and endothelial negative cells (Aiii, Biii). Plots for CD56+ (BV421+ve) and CD56- (BV421-ve) fractions which were collected (Aiv, Biv). FSC-A forward scatter area; FSC-H forward scatter height; SSC-A side scatter area; SSC-H side scatter height. N= 2 biological replicates.
\end{figure}
2.6.2.3 RNA Yield and Quality of Sorted Fresh and Fixed cells

Sorted CD56+ and CD56- fractions as well as unsorted pool from fresh and fixed cells were subjected to RNA isolation using the modified protocol described in materials and methods section 2.5.8. The results showed that all cell fractions and unsorted cell extracts had low RNA yield (ranging from 90 – 400ng) as well as RNA quality (except for 1 unsorted cell from fresh muscle tissue), as indicated by low RIN or lack of RIN reported (Figure 2.12). Consistent with this, the mRNA isolated from most fractions had no clear 18S and 28S bands indicating that it was degraded. These results rendered the samples unsuitable for comparative RNA sequencing analysis as had been intended in this experiment.

Figure 2.12 Assessment of RNA yield and integrity in sorted and unsorted fractions.

The gel image shows the separation profile of each sample along with the RIN. Most of the tested fractions lacked 28S and 18S subunits at 4000 bp and 2000 bp respectively indicating degraded RNA samples.
2.7 Discussion

Skeletal muscle is endowed with a remarkable regenerative potential in response to muscle injury or vigorous physical activity owing to a resident population of stem cells (satellite cells). Primary cultures isolated from skeletal muscle often contain mixed populations of myogenic and non-myogenic cells thus analyses of specific precursor populations requires adequate isolation from muscle extracts (Baquero-Perez et al., 2012).

In this chapter I showed that fresh CD146+ cells isolated from porcine muscle extracts by FACS expressed muscle stem cell markers (PAX7 and CD56) while CD146- cells expressed preadipocyte marker (PDGFRα). In addition, the CD146+ cells showed sole myogenic potential while both the CD146+ and CD146- cells had adipogenic potency in vitro. In addition, I attempted to isolate quiescent muscle stem cells by fixing the cells in situ and showed that while fixation with PFA for short period of time does not alter mRNA yield, composition and quality, the length of time involved in the cell extraction process and subsequent FACS (30hrs) tends to result in significant degradation of mRNA, particularly in tissues that are already fixed with PFA.

FACS have been extensively used to isolate precursor cells from muscle tissue of mice and humans (Liu, Cheung, Charville, & Rando, 2015), significantly aiding in the understanding of myogenic cell commitment, muscle development and disease as well as the development of cell based therapies for muscle disease. However, a lack of universal cell surface markers has hindered the direct translation of established FACS protocols across different species. Previous studies in pig showed that the use of FACS coupled with selection for cell surface markers, CD56 and CD29, can be used to purify myogenic progenitor cells from dissociated muscle tissue but information on the adipogenic potential of the cells was lacking (Ding et al., 2017). In addition, CD56 has been used to isolate myogenic precursor cells from various species including mouse (Capkovic et al., 2008), humans (Pisani et al., 2010c), bovine (Ding et al., 2018) and pigs (Perruchot et al., 2013b).

The choice of CD146 as a prospective marker for isolating myogenic progenitor cell from pig was based on previous data from Donadeu’s laboratory (Figure 2.1), and on previous reports that in addition to being a marker of pericytes/perivascular cells in tissue from various species (Crisan et al., 2008b, Esteves et al., 2017), it has been
successfully used for isolating myogenic progenitor cells from human adult and foetal muscles (Sacchetti et al., 2016, Lapan et al., 2012b, Persichini et al., 2017). My findings that CD146+ but not CD146- cells were able to form myotubes and express Myosin heavy chain and Myogenin when exposed to myogenic conditions indicates that CD146 is effectively a marker of myogenic precursor cells in pigs and is consistent with findings in mouse (Mierzejewski et al., 2020b, Sacchetti et al., 2016) and human foetal skeletal muscle (Cerletti et al., 2006, Lapan and Gussoni, 2012a, Lapan et al., 2012b, Lapan, 2012).

The expression of CD146 or nestin allows for the identification of skeletal muscle pericytes with myogenic potential (Sacchetti et al., 2016, Meng et al., 2011, Birbrair et al., 2013c), although nestin is also expressed in satellite cells (Mierzejewski et al., 2020b). However, the features of pericytes are heavily influenced by their source (Vezzani et al., 2016). For example, muscle derived pericytes and bone narrow derived pericytes are inherently myogenic and skeletogenic respectively but not vice versa (Sacchetti et al., 2016). Moreover, based on the expression of nestin, Birbrair and colleagues described two subpopulations of mouse skeletal muscle pericytes; NG2+/Nestin- (Type I) and NG2+/Nestin+ (Type II) pericytes and only Type II pericytes were able to follow the myogenic program in vitro (Birbrair et al., 2013b, Birbrair et al., 2013a). Similarly, Nirwane et al. (2017) have shown that PDGFRβ+/Nestin- (Type I) and PDGFRβ+/Nestin+ (Type II) pericytes isolated from mouse skeletal muscle are adipogenic and myogenic respectively.

Whether satellite cells and skeletal muscle pericytes are overlapping populations is currently not known. Notably, a recent study, revealed that CD146+ cells from mouse muscle constitute a population of interstitial progenitors with clonogenic and myogenic potential both in vitro and in vivo. However, these cells lacked the expression of PAX7 and CD56 and were therefore thought to be pericytes different from satellite cell-derived myoblasts (Mierzejewski et al., 2020b). Contrary to that finding, human CD146+ myogenic pericytes have been shown to express PAX7 and act like satellite cells in certain in vitro and in vivo assays, suggesting that they may be subsets of the same population that have been arbitrarily recruited to distinct (but close) anatomical sites (i.e. satellite cell niche or perivascular area) (Sacchetti et al., 2016, Persichini et al., 2017). In their study, Birbriar and colleagues showed that 5.6 ± 1.2% of the total cells isolated were NG2+/Nestin- /CD146+/ PDGFRβ+ (type I) while 7.3 ± 1.0% were
NG2+/Nestin+/CD146+/PDGFRβ+ (type II) pericytes corresponding to fibrogenic and myogenic populations respectively. Similarly, CD146+ myogenic cells in my study expressed the satellite cell markers PAX7 and CD56, and the pericyte marker CD146 (which also co-localised with CD56+ cells by flow cytometry), indicating that they constituted a mixed population of pericytes and satellite cells.

In addition to myogenic cells, two adipogenic populations expressing platelet-derived growth factors alpha (PDGFRα) and Spinocerebellar ataxia type 1 have been identified in mouse skeletal muscle (Schulz et al., 2011, Joe et al., 2010b, Uezumi et al., 2010). Uezumi and colleagues showed that PDGFRα is a bona-fide marker of pre-adipocytes in skeletal muscle from mice (Uezumi et al., 2010) and humans (Uezumi et al., 2014b). Similarly, results from my study show that the freshly isolated CD146-cells express PDGFRα, form mature adipocytes and express the lipid carrier protein FABP4 in the presence of adipogenic inducers, indicating that the CD146-cells contain adipogenic progenitor cells. However, CD146+ cells in the present study also displayed adipogenic potential albeit to a lesser extent compared to the CD146- cells. This is not surprising owing to the heterogenous nature of skeletal muscle resident progenitors showing diverse lineage plasticies in addition to myogenic potential (Péault et al., 2007, Pannérec et al., 2013, Dellavalle et al., 2011). As mentioned earlier, only type II (NG2+/Nestin+ or PDGFRβ+/Nestin+) mouse skeletal muscle pericytes were able to undergo myogenic differentiation while type I (NG2+/Nestin- or PDGFRβ+/Nestin-) pericyte differentiated into adipocytes in vitro (Birbrair et al., 2013b, Birbrair et al., 2013a, Nirwane et al., 2017). Taken together, it is likely that CD146+ cells in porcine muscle, as isolated in the present study, contain multiple precursor populations, and that further purification using additional markers will be required to obtain a purely myogenic population as shown in the mice studies above (Birbrair et al., 2013b, Birbrair et al., 2013a, Nirwane et al., 2017).

In agreement with an earlier study, I isolated RNA with good quality and yield from formalin fixed cells (Hrvatin et al., 2014). I showed that the RNA yield and quality was comparable between fixed and fresh cells, and that increasing the concentration of formalin did not have any deleterious effect on the quality and yield of RNA. However, both the standard isolation protocol and in situ fixation of muscle tissue and subsequent FACS resulted in degraded RNA with poor yield. Similar to my findings, Machado et al. (2017) reported that RNA quality was not affected when muscle stem
cells were fixed for a short period of time before RNA isolation but showed reduced RNA quality when the muscle tissue was fixed prior to cells isolation and FACS, albeit they reportedly managed to sort the fixed and profile the transcriptome of quiescent SCs.

The RNA degradation observed in Machado’s and my study may be attributed to the cell isolation process as well as length of time taken from cell isolation to RNA extraction. Machado et al. (2017) compared the transcriptomes between cells sorted with or without prior in situ fixation, and showed that a large number of transcripts involved in cell cycle activation were downregulated in the fixed cells compared to the non-fixed cells during the isolation process suggesting that RNA degradation mechanisms may operate at the early phases of satellite cell activation. Similar transcriptional changes were observed during the isolation and purification of muscle stem cells in other studies using muscle stem cell-specific labeling of RNA (van Velthoven et al., 2017a) as well as single cell analysis of satellite cells (van den Brink et al., 2017). This implies again that the length of time taken from cell isolation from muscle tissue to RNA extraction is key in obtaining good quality RNA and may account for the poor RNA quality obtained in my study. Cell extraction, staining, FACS and subsequent sample preservation for RNA analysis took approximately 30 hours in my study compared to 3 hours in Machado’s study using transgenic Tg:Pax7-nGFP mice whose muscle stem cells express nuclear GFP. Therefore, to isolate quiescent muscle stem cells in pigs will require further optimisation of the various steps involved with an aim of drastically shortening the processing time.

2.8 Conclusion

This study demonstrated that CD146 can be used to isolate porcine skeletal muscle progenitor cells. Porcine CD146+ cells expressed PAX7 and CD56, and were able to follow both myogenic and adipogenic programmes in vitro, thus representing multipotent progenitor cells that could include both satellite cells and pericytes. Furthermore, porcine CD146 shared at least two properties (ability to undergo myogenic and adipogenic differentiation in vitro) with previously described multipotent CD146+ pericytes/perivascular cells from multiple human tissues (Crisan et al., 2008b). These cells may provide a useful invitro platform to study the dymanic cross
talk between muscle and fat cells during the development of pig muscle and fat tissues. However, the conditions necessary to differentiate the cells simultaneously into myogenic and adipogenic lineages needs to be optimised. My study also highlights the difficulty of obtaining quiescent SCs from pigs or other livestock species. I show that RNA yield and quality are not altered by brief fixation of cells with PFA but cell extraction (involving many hours) coupled with FACS, with or without fixation, significantly reduces RNA yield and quality. This data provides a foundation for future work toward optimising the methodology for isolating quiescent SCs with high-quality RNA after in situ fixation of muscle tissue in livestock species.
Chapter 3 Derivation and Characterization of Porcine Muscle-Derived Progenitor Cells
3.1 Introduction

Skeletal muscle serves a multitude of functions in an organism and is composed of muscle fibres or myofibres that exhibit different metabolic and contractile properties. These myofibres are formed in a process known as myogenesis (Bentzinger et al., 2012). Skeletal muscle is distinguished by its remarkable capacity for regeneration owing to the presence of satellite cells which though normally quiescent become activated in response disease, injury or exercise. Upon activation these satellite cells proliferate rapidly to form myoblasts which then differentiate and fuse either with one another or with pre-existing damaged myofibres to form multinucleated myofibres, regenerating the damaged muscle (Rudnicki et al., 2008, von Maltzahn et al., 2013, Sacco et al., 2008).

Satellite cells are the tissue resident muscle stem cells, however, they make up only a small fraction of mononuclear cells in skeletal muscle tissue (Tedesco et al., 2010). Instead, muscle regenerative capacity depends on the balanced action of multiple types of different resident progenitor cells including satellite cells, muscle side population, non-satellite myogenic progenitor cells, mesenchymal stem cells, fibro/adipogenic precursor cells as well as pericytes, as reported in previous studies (Klimczak et al., 2018), and which are necessary to produce the wide array of tissues, i.e. myofibres, neurons, blood vessels, adipose and connective, contained within normal skeletal muscle (Zammit and Beauchamp, 2001, Yin et al., 2013).

The ability to isolate and culture muscle cell fractions containing satellite cells and other stem/progenitor cell types has provided immense insight onto the complexities of skeletal muscle growth, regeneration, aging as well as disease (Bareja and Billin, 2013, Shahini et al., 2018). An important challenge to studying the biology and biomedical potential of satellite cells is their propensity, once they are taken from their natural niche and maintained in vitro, to rapidly differentiate into myoblasts, a more committed muscle precursor irreversibly expressing MYF5, MYOG and MYOD (Boonen and Post, 2008). Moreover, obtaining sufficient numbers of functional muscle stem cells is essential for the success of many applications, including tissue engineering, drug screening and cell therapy (Wang et al., 2014).

In addition to the agricultural importance of the pig as the most consumed meat-producing species globally, there is a growing interest in using pigs as animal models
for many biomedical studies due to similarities in porcine organ physiology with human beings (Swindle et al., 2012). Pigs are a preferred animal model for testing stem cell-based therapy, regenerative medicine, and transplantation (Ringe et al., 2002). Therefore, being able to isolate satellite cells, and other myogenic progenitor cell populations from porcine skeletal muscle can provide an important tool for understanding muscle biology, disease and regeneration, and for improving meat production. As mentioned earlier, the variety of muscle progenitor cells is wide. In the same light, studies have suggested that satellite cells exist as a heterogeneous population of committed muscle stem cells (Motohashi et al., 2014). It is, therefore, important to develop protocols enabling selective purification of muscle stem cells. In addition, characterising the progenitor cells obtained from skeletal muscle will help distinguish between the different populations and provide information for their further utilisation.

For the isolation of satellite cells, their progeny myogenic precursor cell (MPCs) also known as myoblasts) and other myogenic progenitor cell, two main methods are currently utilised, namely, enzymatic digestion followed by cell purification, and explant culture. The first, more popular, approach involves enzymatic digestion and trituration of skeletal muscle to obtain a mononuclear cell population. Subsequent purification involves pre-plating, density gradient centrifugation, Fluorescence-activated cell sorting (FACS) or magnetic activated cell sorting (MACS). Both FACS and MACS are considered the most robust methods (Hindi et al., 2017a, Danoviz and Yablouka-Reuveni, 2012a) (Reviewed in chapter 2). Although FACS has been used to successfully purify myogenic cells, one major drawback of this method is the lack of universal surface markers, which limits its use in species other than rodents and humans. In addition, the cell sorting process itself may impair cell survival and proliferation (Beliakova-Bethell et al., 2014, Fong et al., 2009), as a result of the effects of electrical currents used to separate the cells (Balda and Matter, 2009) or of cell surface-binding antibodies in activating or blocking signalling pathways associated with target cell receptors (van Velthoven et al., 2017b). Similarly, since MACS depends on surface marker binding, it suffers from some of the same drawbacks as FACS including the lack of universal surface markers. In addition, when used as a positive selection method, the attachment of magnetic microbeads may hinder downstream utilisation of these cells (Servida et al., 1996), for example the use of cells with
attached or internalized microbeads for tissue engineering applications. Overall, both purification methods can be expensive and time consuming. In addition, purity of cells obtained is variable often resulting in an overgrowth by fibroblasts within a week of culture (Benedetti et al., 2021, Syverud et al., 2014, Keire et al., 2013).

The second method for isolating satellite cells involves culture of single fibres or pieces of muscle tissue to allow cells to migrate out of the tissue during incubation (ASKANAS and ENGEL, 1975, Smith and Schofield, 1994). This method was originally used to isolate and culture live single fibres from rat muscle with the aim to create an \textit{in vitro} system that maintained the physical association between the muscle fibre and its stem cells (Bischoff, 1986). The method has since gained popularity and has been adapted to different muscles from different species, age or conditions (Au - Pasut et al., 2013, Shefer and Yablonka-Reuveni, 2005, Rosenblatt et al., 1995, Anderson et al., 2012, Shahini et al., 2018, Wang et al., 2014, Marg et al., 2020). The most obvious advantage of the explant method is that it minimises the trauma to myogenic progenitor cells associated with enzymatic digestion and subsequent purification. In addition, the action of cutting up muscle tissue samples into smaller pieces for explanting by itself mimics muscle trauma thereby triggering satellite cell activation, migration, and proliferation (Harvey et al., 1979, Smith and Schofield, 1994), making explant culture a good mimicry of the \textit{in vivo} environment. Also, since the dissection process mimics tissue injury it results in the release of soluble factors as well as growth factors from emerging myoblasts. These factors provide conditioned media that supports the survival and proliferation of skeletal muscle stem cells (Smith and Merrick, 2010).

The activity of muscle stem cells is regulated through multiple extrinsic cues within the ‘niche’ which comprises the basement membrane of the myofibres, supporting cells and the local extracellular matrix (ECM) proteins (reviewed by (Wang et al., 2014)). The ECM proteins provide attachment site and growth factors for the satellite cells and include collagens, laminin, fibronectin and heparin sulphate proteoglycans (HSPGs) (Boonen and Post, 2008).

Isolation and culturing of muscle stem cells often leads to a change in their characteristics manifested as a reduction in their proliferation, migration, differentiation, and regenerative potential. However, including some of these ECM
components may serve as extrinsic cues *in vitro* to help to enrich the muscle stem cell population, provide support for cell adhesion while maintaining stem cell myogenic and self-renewal properties (Rodin et al., 2010, Melkoumian et al., 2010, Villa-Díaz et al., 2010). ECM components are commonly incorporated into cell culture conditions as substrates for coating tissue culture flasks prior to culturing of cells. The commonly used matrices are laminin, collagen, gelatin and matrigel. While collagen and gelatin are relatively cheap and easy to use, matrigel and laminin coatings are the preferred coatings as the myotubes formed on the previous matrices are shorter than those formed on the later (Grefte et al., 2012, Wilschut et al., 2010a). In addition, coating with matrigel, was found to produce a significantly larger pure population of MPCs, sustain muscle stem cell proliferation, improve muscle stem cell differentiation as well as preservation of their myogenic regeneration capabilities (Wilschut et al., 2010a, Danoviz and Yablonka-Reuveni, 2012b, Vaughan and Lamia, 2019, Wang et al., 2014, Shahini et al., 2018). Matrigel is composed of different ECM proteins and contains approximately 60% laminin, 30% collagen IV, and 8% entactin as well as heparan sulfate proteoglycans and growth factors suggesting that a balanced mixture of ECM components could be more efficient for proper muscle differentiation compared to single protein substrates (Shahini et al., 2018).

Muscle stem cell populations have been much less characterised in pig than in human and mouse. Several approaches have been reported to purify porcine muscle stem cells including pre-plating, percoll density gradient centrifugation, FACS and MACS (Choi et al., 2020a, Ding et al., 2017, Wilschut et al., 2010b, Mau et al., 2008, Perruchot et al., 2012, Doumit and Merkel, 1992, Mesires and Doumit, 2002). However, the cell identity of the stem cell populations obtained by the above methods, as well as their long term multi lineage differentiation capacity have not been carefully characterized (Reviewed in Chapter 2). Moreover, despite the popularity of the explant method of isolation of muscle stem cells, only one study have reported using this method of isolation for pig stem cells where muscle progenitor cells were induced to migrate from protease-digested muscle tissue fragments on gelatin coated flasks. While the progenitor cells obtained showed a long term expansion, expressed mainly MSC markers and could differentiate into adipogenic and osteogenic lineages, they did not express muscle stem markers and failed to form myotubes *in vitro* except when
co-cultured with murine C2C12 myoblasts, indicating that these cells were not myogenic progenitors (Wilschut et al., 2008).

3.2 Objective

The main objective of the study presented in this chapter was to develop and characterise an explant-based culture model for the isolation and maintenance in vitro of muscle stem cells from pigs. For description purposes, the cells will be termed muscle derived progenitor cells (MDPCs) and referred to as thus for the rest of the thesis.

3.3 Experimental Aims

The specific aims include;

1. To develop a robust method for enrichment of porcine myogenic precursor cells using the explant method.

2. To determine their proliferative capacity during long term expansion in vitro.

3. To determine the phenotypic identity of the cells during long-term expansion in vitro by measuring their expression of key lineage markers.

4. To determine the plasticity of the MDPCs through their ability to differentiate into myogenic and adipogenic lineages during long term expansion in vitro.
3.4 Materials and Methods

3.4.1 Isolation and Culture of Muscle Derived Progenitor Cells (MDPCs).

Semitendinosus muscle was obtained from four Landrace piglets, four weeks old, following euthanasia with intravenous injection of sodium pentobarbitone 20% w/v (Henry Schein Animal Health, Dumfries, UK). Once death was confirmed, the skin and connective tissues of the hind limb were removed to expose the musculature and the semitendinosus muscle (shown in Figure 2.3) was dissected aseptically. Muscle samples (1-2g of muscle tissue) were immediately transferred to phosphate buffered saline (PBS) supplemented with 2.5 µg/ml Amphotericin B (Gibco, ThermoFisher Scientific, cat #15290-026), and 1% penicillin-streptomycin (PS) (Gibco, ThermoFisher Scientific, cat #1540-122) (PBS+F+PS solution) and transported on ice to the laboratory for cell isolation and culturing.

Once in the lab, muscle tissue was used to prepare the explant culture following a protocol adapted from Shahini et al. (2018). First the piece of muscle tissue was washed in three changes of cold PBS+F+PS solution to remove any debris. Next the muscle tissue was carefully dissected to remove fat and connective tissue and subsequently minced into small muscle fragments. These muscle fragments were washed again in 2 changes of cold PBS+F+PS prior to plating on matrigel-coated 6 well tissue culture plates.

Matrigel (10 mg/ml, BD Biosciences, cat #354234) coating was done at room temperature with matrigel diluted 50x in cold Dulbecco's Modified Eagle Medium (DMEM) high glucose (Life Technologies, cat #41966029) and incubated at 37°C. After 30 min, matrigel solution was removed and fresh proliferation media (Hams F10 nutrient mix (Life Technologies, Thermo Fisher Scientific, cat # 31550023) supplemented with 20% Foetal Bovine Serum (FBS, Life Technologies, Thermo Fisher Scientific, cat #10500064), 1% PS, 2.5 µg/ml amphotericin B and 5 ng/ml basic fibroblast growth factor (bFGF, PeproTech, London, UK, cat # 100-18B)) was added to the wells. Then the muscle fragments were carefully transferred to the wells containing media and incubated at 39°C with 5% CO₂. The flaskes were allowed to sit for 2 days to allow attachment of the tissue and subsequent egression of cell. Cells were observed every other day carefully under a brightfield microscope and fresh media added as needed. After a maximum of 14 days in culture, tissue fragments were
removed and cell monolayers washed with PBS prior to detaching the cells with 0.25% Trypsin-EDTA (Fisher Scientific UK, cat#25300-054). The isolated cells were either frozen in freezing media (5% DMSO; 95% FBS) or seeded for expansion and further experiments.

3.4.2 Cell Proliferative Assay

The MDPCs were expanded serially by replating trypsinised cells on tissue culture flasks coated with 0.1% gelatin (Merck Life Science, UK. Cat# 9000-70-8) at a density of 5000 cells/cm² in proliferation media and incubated at 39°C at 5% CO₂. Cells were maintained at a density of < 70% confluence and counted at each passage. To count the cells, 10 µl of cell suspension was mixed with 0.4% Trypan Blue solution (Sigma Aldrich: cat # T8154) in a 1:1 dilution and 10 µl of the resulting diluted cell suspension is pipetted onto the haemocytometer chamber. The cells in the chamber were viewed under the Nikon Eclipse TS100 inverted microscope. Dead cells appeared blue as they take up trypan blue and were excluded from the count.

The doubling time of the cells were calculated at each passage using the formula:

\[
\text{Doubling Time} = \frac{\text{Time in culture} \times \log(2)}{\log(\text{Final number}) - \log(\text{Initial number})}
\]

Where Initial number refers of cells seeded, and final numbers refers to number of cells at the time of harvesting.

3.4.3 Flow Cytometry Analysis of MDPCs

To characterise the porcine MDPCs by flow cytometry, the cells were dissociated into single cells, washed in PBS and resuspended in FACS buffer (1% Bovine serum albumin, BSA in PBS) to a final dilution of 0.5*10^6/ml. Depending on the antibody being investigated, non-specific binding was blocked by incubation with either 10% mouse serum (Sigma Aldrich, cat# M5905-10ML) or 10% goat serum (Abcam, cat# ab7481) in PBS at 4°C for 30 min. Subsequently, the cells were incubated for 1 hour at 4°C with antibodies as shown in table 3.1.
Table 3.1 List of Antibodies and the respective isotype controls used for flow cytometry.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Catalogue Number</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD29-AF700</td>
<td>BioLegend</td>
<td># 303020</td>
<td>1:25</td>
</tr>
<tr>
<td>CD31-RPE</td>
<td>AbD Serotec-BioRad</td>
<td># MCA1746PET</td>
<td>1:13</td>
</tr>
<tr>
<td>CD45-FITC</td>
<td>AbD Serotec-BioRad</td>
<td># MCA1222F</td>
<td>1:20</td>
</tr>
<tr>
<td>CD56-BV421</td>
<td>BD Biosciences</td>
<td># 562751</td>
<td>1:50</td>
</tr>
<tr>
<td>CD56-FITC</td>
<td>BioLegend</td>
<td># 304603</td>
<td>1:20</td>
</tr>
<tr>
<td>CD90-BV605</td>
<td>BD Pharimgen</td>
<td># 747750</td>
<td>1:100</td>
</tr>
<tr>
<td>CD140a-PE</td>
<td>BioLegend</td>
<td># 323505</td>
<td>1:25</td>
</tr>
<tr>
<td>CD146-AF647</td>
<td>AbD Serotec-BioRad</td>
<td># MCA2141A647</td>
<td>1:50</td>
</tr>
<tr>
<td>CD105 (purified)</td>
<td>Abcam</td>
<td># ab69772</td>
<td>1:40</td>
</tr>
<tr>
<td>Anti-mouse IgG2a-APC</td>
<td>BioLegend</td>
<td>cat# 407110</td>
<td>1:200</td>
</tr>
</tbody>
</table>

**Isotype controls**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Catalogue Number</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse IgG2a, k (Purified)</td>
<td>Abcam</td>
<td># ab18415</td>
<td>1:40</td>
</tr>
<tr>
<td>Mouse IgG1 AF647</td>
<td>AbD Serotec-BioRad</td>
<td># MCA928A647</td>
<td>1:50</td>
</tr>
<tr>
<td>Mouse IgG1, κ AF700</td>
<td>BioLegend</td>
<td># 400144</td>
<td>1:25</td>
</tr>
<tr>
<td>Mouse IgG2b, k BV421</td>
<td>BD Biosciences</td>
<td># 562748</td>
<td>1:50</td>
</tr>
<tr>
<td>Mouse IgG1, κ BV605</td>
<td>BioLegend</td>
<td># 562652</td>
<td>1:100</td>
</tr>
<tr>
<td>Mouse IgG2a, κ FITC</td>
<td>AbD Serotec-Biorad</td>
<td>#MCA928F</td>
<td>1:20</td>
</tr>
<tr>
<td>Mouse IgG1 RPE</td>
<td>BD Pharimgen</td>
<td>#MCA928</td>
<td>1:13</td>
</tr>
</tbody>
</table>

After 1 hour, the cells were washed 3 times with FACS buffer and unconjugated Anti-CD105 was further incubated with APC labelled secondary antibody for 30 min. Isotype controls shown in table 3.1 were used at same concentrations as their respective primary antibodies and analysed in parallel.

To determine cell viability the samples were further incubated with Zombie Aqua viability dye (1000x dilution, BioLegend, cat # 423101) for 15 min at room temperature in the dark and washed once in FACS buffer prior to analysis. Flow analysis was
performed on BD Fortessa X20, and data analysed with FACSDiva software (BD Biosciences, San Jose, CA, USA) or FlowJo (LLC, Ashland, OR, USA).

3.4.4 RNA Extraction and Gene Expression Analysis

For each experiment, samples were collected in Trizol and RNA extracted from these samples as described in section 2.5.7. Using 0.5 µg per test sample, total RNA was reverse transcribed in a 2-step reaction using the SuperScript III RT kit (Invitrogen, cat #18080-044) and quantitative real time polymerase chain reaction (qRT-PCR) was performed using Sensi-FAST SYBR Lo-ROX Kit (Bioline UK, cat #BIO-94050) as described in section 2.5.9. All samples and controls were run in duplicates and the results generated analysed on the MxPro software and excel spreadsheet. All samples were normalized against two reference genes (18s and RPL4). The primers sequences used for gene analysis are listed on Table 2.1.

3.4.5 Immunofluorescence Analysis of MDPCs

Immunostaining for MPDCs was done as described in section 2.5.10 with a few changes. Following fixation, permeabilisation of the MDPCs was done in either 1:1 methanol: acetone solution (for surface antibodies, 10min) or 0.5% Triton-X-100 (for nuclear antibodies; 15 min) at room temperature. Next, the MDPCs were blocked for nonspecific antibody binding in either protein block solution (Abcam, cat #ab64226; MYHC) or homemade blocking solution (10% goat serum, 2% BSA, 0.25% Triton® X-100 in 1X PBS; nuclear antibodies) at room temperature for 1 hour or 30 min respectively. Subsequently, the primary antibody (anti Pax7, 5 µg/ml: R&D laboratories, Huntingdonshire, UK cat# MAB1675 ; anti-MYOD: 20 µg/ml, Agilent Technologies, Cheshire, UK; cat #M351201; anti-MYHC 5 µg/ml, R&D laboratories, Huntingdonshire, UK; cat #MAB4470) was incubated for 16hr at 37°C (PAX7) or 4°C overnight (MYOD and MYHC) in blocking solution (MYOD and PAX7) or antibody diluent reagent (for MYHC, ThermoFisher Scientific, Uk; cat #003118). After washing the MDPCs in PBS or PBST (3times, 5min) to remove any unbound primary antibody, secondary AF488-conjugated goat anti-mouse antibody incubation for 1 hour at RT was performed. Cells were washed in PBS or PBS plus 0.05% Triton X (PBST) for a
further 3 times (5min each) and overlayed with a mountant containing 4′,6-Diamidino-2-phenylindole (DAPI) (Fluoroshield with DAPI; Sigma, cat #F6057) to counterstain the nuclei and subsequently covered with a coverslip. Images were captured on an Axiovert 25 and Axiovert 200M inverted microscopes and analysed on the Zen Software (Zeiss, Oberkochen, Germany).

3.4.6 Muscle Differentiation

The MDPCs were primed in proliferation media (80 nM dexamethasone; Sigma Aldrich; cat# D4902; 10% FBS and 1% PS) and myotube formation was induced in serum free medium supplemented with 1% Insulin-Transferrin-Selenium (100X, Gibco-Thermofisher scientific, cat# 41400045) and 1% PS for 5 days as described in section 2.5.5. Samples were taken at days 0, 3 and 5 in TRIZol reagent and stored at -80°C for gene expression analysis detailed in 2.5.9 while some wells were fixed in 4% PFA for immunocytochemistry on day 5.

3.4.7 Adipogenic Differentiation

The MDPCs were induced to differentiate in adipose differentiation media and adipose maintenance media as described in section 2.5.7 for 14 days. Samples were taken at days 0, 7 and 14 and were harvested in TRIZol for RNA extraction and gene expression analysis (details in section 2.5.9). Subsequently, adipocytes were visualised by staining with Oil Red O (ORO; Sigma-Aldrich, cat# 00625) as detailed in section 2.5.11 and Images were captured in a Zeiss Axiovert 25 inverted microscope.

3.4.8 Statistical Analysis

All Graphs were produced using GraphPad PRISM software (version 9.0.2; La Jolla, CA USA) with data presented as mean ± SEM. Statistical analyses were performed using Minitab 20 Statistical Software (2022) (Computer software, Pennsylvania, USA). Data were assessed for normal distribution using the Kolmogorov–Smirnov test (P<0.01) and log-transformed before further analyses if not normally distributed. Unless otherwise stated, statistical significance was determined using one-way or two-
way ANOVA and a subsequent comparisons among means with Tukey’s test with statistical significance set at P<0.05.
3.5 Results

3.5.1 Derivation of Porcine MDPC Cultures

Semitendinosus muscle was obtained from 4 weeks old pigs, minced finely into small muscle fragments without enzymatic digestion and seeded on matrigel-coated dishes. These explants were cultured in vitro in proliferation media to allow cells to egress from the muscle tissue. After 10-14 days, the muscle explant was removed, the cells washed and passaged onto fresh tissue culture flasks. After 4 days of explant culture, cells were observed to egress from the skeletal muscle explant and proliferate (Figure 3.1A-C). At this stage, most of the outgrowing cells appeared to be small and compact and were most likely satellite cells (Figure 3.1A-B, white open arrowhead). After, passaging, most of the cells had small and spindled- shaped morphology with single nuclei and were most likely activated myoblasts (Figure 3.1D, white closed arrowhead). A few of the cells appeared flat and elongated and were most likely fibroblasts (Figure 3.1D, white arrows). To analyse the proliferation ability of the outgrown cells, they were serially passaged at a low density (5*10^3 cells/cm2) in proliferation media for up to 50 days (14 passages). During this time, the MDPCs maintained an average doubling time of 2.23 ± 0.27 days; their doubling time increased from 1.42 ± 0.16 days in passage 1 (p1) to 2.41 ± 0.02 days in p14 (Figure 3.1E; P<0.0001; n= 4 biological replicates) indicating a reduction in growth rate of porcine MDPCs in vitro.
Figure 3.1 Porcine MDPCs derivation and culture.

(A to C) Egression of cells from skeletal muscle tissue (SKM) at 4, 6 and 10 days after seeding explants in matrigel-coated cell culture plates. Scale bars, 200µm in A, B, and 400µm in C. (D) Representative phase contrast image showing the morphology of MDPCs after 1 passage (p1) in culture. Scale bar, 200µm. (E) Rate of MDPCs proliferation shown as doubling times (in days) over passages 1-14. Data shown are mean ± S.E.M, n=4 biological replicates. P>0.001. Means for data points with different subscripts are significantly different (P<0.05)
Figure 3.2 Characterization of expanded MDPCs.

(A) Relative mRNA levels of muscle stem cell markers PAX7, MYOD and MYF5 in MDPCs at different passages (p). Data are shown as mean ± S.E.M. Means with different superscripts are different (P<0.05). (n=4 independent experiments).
3.5.2 Expression of Lineage-specific and Cell Surface Marker by Porcine MDPCs

MDPCs from pig skeletal muscle explants were characterised by analysing changes in the expression of different myogenic and mesenchymal progenitor markers, by RT-qPCR and immunofluorescence and/or flow cytometry, following serial passaging in culture.

3.5.2.1 Myogenic Markers

Mean mRNA levels of the lineage-specific transcription factors, PAX7, MYOD and MYF5, were relatively stable in MDPCs during early passaging, and started to decrease progressively after p2 and up until p14 (Figure 3.2A-C). Consistent with this, immunostaining for PAX7 and MYOD were dramatically reduced in MDPCs at p6 and p10 relative to p2 (42.3 ± 3.8 % vs 3.4 ± 0.4% vs 0.33 ± 0.1 % at p2, p6 and p10 respectively; P>0.001) for PAX7 (Figure 3.3A-B) and (46.1 ± 6.1% vs 2.4 ± 0.4% vs 1.7 ± 0.9% at p2, p6 and p10 respectively, P>0.001 for MYOD (Figure 3.4A-B). Representative Immunostaining for cell outgrowths at p0 (i.e 7 days after explanting and before cells were passaged) is shown in Figure 3.3A top lane and 3.4A, top lane. Moreover, immunocytochemical analyses of these p0 cells showed that 23.5 ± 8.0 % and 30.95 ± 4.07% of cells stained positive for PAX7 and MYOD, respectively, comparable (P>0.1) to levels in p2 cells (42.3 ± 6.6 % and 46.1 ± 6.1% for PAX7 and MYOD respectively). Unstained cells, an IGG negative control specific for the species of the primary antibodies, and a secondary control (antibody only) were used as controls.

mRNA levels of the cell surface marker, neural cell adhesion molecule (NCAM), also known as CD56, did not change significantly with serial passaging (Figure 3.5A). In addition, the expression of CD56 was analysed by flow cytometry. A distinct peak was observed for antibody positive cells which was different from the peak produced by the isotype control showing that, at all passages investigated, the majority of the cells sampled expressed CD56 (Figure 3.5B). However, the fluorescence intensity appeared to decrease with serial passaging, as indicated by a shift to the left of the positive peak on the X-axis (Figure 3.5B), while the mean fluorescence intensity (MFI) tended to decrease (6529.9 ± 1174; 3674 ± 912 and 1944.6 ± 472.6 at p2/3, p6/7 and
p10/11, respectively, P=0.16) (Table 2.2). This suggests that antigen levels likely differ between cells types present in the pool of samples.
Figure 3.3: Characterization of expanded MDPCs.

(A) Representative immunofluorescent PAX7 staining (green) of porcine MDPCs at p0 (i.e. day 7 of muscle explant and before passaging) and at passages 2, 6 and 10; blue shows DAPI staining. Scale bars, 100µm. (B) Quantitative immunofluorescent staining of PAX7 in MDPCs at passages 2, 6 and 10 (n=3 independent experiments). Data are shown as mean ± S.E.M. Means with different superscripts are different (P<0.05).
Figure 3.4 Characterization of expanded MDPCs

(A) Representative immunofluorescent MYOD staining (green) of porcine MDPCs at p0 (i.e. day 7 of muscle explanting before passaging) and at passages 2, 6 and 10; blue shows DAPI staining. Scale bars, 100µm. (B) Quantitative immunofluorescent staining of MYOD in MDPCs at passages 2, 6 and 10 (n=3 independent experiments). Data are shown as mean ± S.E.M. Means with different superscripts are different (P<0.05).
Figure 3.5 Characterization of expanded MDPCs.

(A) Relative mRNA levels in MDPCs as measured by qRT-PCR of myogenic cell surface marker CD56 across different passages in culture. Data shown are mean ± S.E.M, n=4 independent experiments. (B) Flow cytometry analysis of CD56 surface marker in MDPCs at p2/3, p6/7 and p10/11. Representative flow cytometry histograms showing percentages of MPCs positive (light grey displaced to the right) for CD56 Ncam16.2 at the 3 passages investigated. Signal from the corresponding isotype controls is shown in dark grey displayed on the left. Number of cells positive for CD56 is shown in graph.
3.5.2.2 Mesenchymal Progenitor Markers

RT-PCR analyses of the adipocyte progenitor marker, Platelet-Derived Growth Factor Receptor Alpha (PDGFRα), showed a significant increase with serial passaging (Figure 3.6A; P<0.01). Likewise, mean levels of CD105 mRNA increased progressively during serial passaging (Figure 3.6B; P<0.01), whereas changes in CD90 and CD44 mRNAs were not significant (Figure 3.6 C-D; P>0.1).

Changes in the expression of PDGFRα, CD105 and CD90 across passages were also quantified using flow cytometry. The percentage of cells expressing PDGFRα increased, on average, across p2, p6 and p10, however this did not reach significance (Figure 3.7A; p>0.1). Likewise, mean MFI values were not different among passages (Table 2.2). For CD105 and CD90, consistent with RT-qPCR data, there was an increase in the mean percentage of CD105 positive cells during serial passaging (Figure 3.7B; p< 0.05) but no differences in CD90 (Figure 3.7C, P>0.1). In addition, MFI values did not change with passaging for either CD105 or CD90 (Table 2.2).

The expression profile of CD146 in serially passaged MDPCs was also analysed by flow cytometry. Percentage of CD146-positive cells decreased (Figure 3.7D, P<0.05) while the MFI tended to decrease with passaging (Table 2.2; P=0.068).

Finally, CD31 was also detected in MDPCs although its levels did not change during culture (59±16%, 91.3±3 and 60.6±16.2 at p2/3, p6/7 and p10/11 respectively). In contrast, CD45 staining was not detected, suggesting the presence of endothelial but not hematopoietic cells in those cultures.
Figure 3.6 Characterization of expanded MDPCs.

(A) Relative mRNA levels of mesenchymal progenitor markers PDGFRα, CD105, CD90 and CD44 in MDPCs at different passages. Data are shown as mean ± S.E.M. Means with different superscripts are different (P<0.05). n=4 independent experiments). P= passages
Figure 3.7 Flow cytometry analysis of PDGFRα, CD105, CD90 and CD146 in MPDCs at different passages.

Representative flow cytometry histograms showing percentages of MDPCs positive (light grey displaced to the right) for PDGFRα (A), CD105 (B), CD90 (C) and CD146 (D) at the 3 passages investigated. Signal from the corresponding isotype controls is shown in dark grey displayed on the left and percentage of positive cells is shown in the graph. Data are shown as mean ± S.E.M. Means with different superscripts are different (P<0.05). n=3 independent experiments). P= passages.
Table 3.2 the mean fluorescent intensity (MFI) of various markers measured by flow cytometry in porcine MDPCs at p2, p6 and p10.

<table>
<thead>
<tr>
<th>Marker</th>
<th>p2/3</th>
<th>p6/7</th>
<th>p10/11</th>
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<tbody>
<tr>
<td>CD56 Ncam 16.1</td>
<td>6529.9 ± 1174</td>
<td>3674 ± 912</td>
<td>1944.6 ± 472.6</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>228 ± 84</td>
<td>280 ± 79</td>
<td>190.1 ± 48.6</td>
</tr>
<tr>
<td>CD105</td>
<td>1016 ± 100</td>
<td>2291 ± 914</td>
<td>1881.1 ± 237.8</td>
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<tr>
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<td>4440 ± 1146</td>
<td>2994.3 ± 826.7</td>
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<tr>
<td>CD146</td>
<td>1245 ± 186</td>
<td>568 ± 187</td>
<td>547.0 ± 244.7</td>
</tr>
<tr>
<td>CD31</td>
<td>1103 ± 643</td>
<td>2245 ± 1285</td>
<td>2146.0 ± 1302.3</td>
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<tr>
<td>CD45</td>
<td>23 ± 7</td>
<td>15 ± 0</td>
<td>24.9 ± 6.4</td>
</tr>
</tbody>
</table>

Data shown as mean ± standard error of the mean for each marker measured. p= passage

3.5.3 Differentiation Capacity of Porcine MDPCs

3.5.3.1 Myogenic Differentiation

The ability of MDPCs to form myotubes was assessed at p1, 2, 4, 6, 8 and 12. When placed under differentiation conditions, porcine MDPCs produced visible multinucleated myotubes at all passages except for p12 (Figure 3.8A). Their differentiation efficiency was noticeably reduced after p4 as indicated by thinner and fewer myotubes. Consistent with these observations, mRNA levels of MYOG, as well as adult and developmental myosin heavy chain isoforms (MYH1 and MYH3, respectively) in differentiated cells progressively decreased with passage number (Figure 3.8 B-C)
Figure 3.8 Characterisation of expanded MDPCs in Myotube formation.

(A) Bright field images showing myotube formation in passages 1 (p1), 2 (p2), 4 (p4), 6 (p6) and 8 (p8) but no myotubes seen at 12 (p12) of MDPCs. Scale bars, 200µm. Images taken on day 3 after induction in differentiation medium. (Insert) Representative image showing Immunostaining of differentiated myotubes for myosin heavy chain at p1, (MYHC: green; Dapi: blue). Scale bar, 50µm. (B-D) Relative mRNA levels as measured by qPCR of MYOG, MYH1, and MYH3 transcripts on day 3 of differentiation at different passages. The values are displayed as a fold change expression relative to the values on Day 0. Data shown are mean ± S.E.M, n=3 independent experiments. Means that do not share a letter are significantly different.
3.5.3.2 Adipogenic Differentiation

Adipogenic ability was determined in cells at p2, 6 and 14. After 14 days in adipogenic media, cells at p2 remained elongated and did not show lipid accumulation (Figure 3.9A, left panel). However, at passage 6 and passage 14, cells became rounded and displayed visible lipid droplets (Figure 3.9A, middle and right panel) as confirmed by oil-red-oil staining (Figure 3.9B). Analyses of adipogenesis associated genes confirmed the above findings as indicated by an increase in the expression fatty acid carrier protein (FABP4) and the transcription factor, peroxisome proliferator-activated receptor gamma (PPARγ) mRNA at P6 and P14 (Figure 3.9C-D). Taken together, the porcine MDPCs lack adipogenic potential at the early passage but acquire this following serial passaging.
Figure 3.9 Characterisation of expanded MDPCs in adipogenic differentiation.

(A) Representative bright-field images of MDPCs at different passages cultured in adipose differentiation conditions for 14 days. No adipocyte formation in porcine MDPCs at p2, intracellular lipid droplets at (p6) and (p14). Scale bar = 200μm. (B) Image showing oil red O staining for intracellular lipid accumulation. Scale bar = 50μm. (C-D) Relative mRNA levels in MDPCs of adipogenic regulatory genes FABP4 (C) and PPARγ (D) as measured by qPCR at p2, p6 and p14. The values are displayed as a fold change expression relative to the values on Day 0. Data shown are mean ± S.E.M, n=4 independent experiments. Means with different superscripts are significantly different (P<0.01).
3.6 Discussion

Robust isolation, expansion and long-term \textit{in vitro} maintenance of muscle progenitor cells from livestock species would provide a highly valuable tool for understanding muscle development in those species towards improving the efficiency of animal meat production as well as potentially benefiting the emerging area of cell-based foods. The present study provides a step forward by reporting the successful derivation and detailed characterisation of a novel tissue explant-derived culture system in porcine allowing long-term maintenance of MDPCs, and which provides several advantages over the classic use of enzyme-based cell isolation methods in this species. The method takes advantage of the ability of satellite cells to become activated upon injury (induced by mechanical mincing in this case), migrate and proliferate rapidly to repair the damaged tissue (Pasut et al., 2013b, Wang et al., 2014, Shahini et al., 2018, Smith and Meyer, 2020). The MDPCs obtained from this method showed long-term expansion \textit{in vitro}, expressed key muscle stem cell markers and were able to efficiently form myotubes over several passages \textit{in vitro}, eventually losing their myogenic potential and acquiring adipogenic potential with prolonged culturing.

Muscle explants gave rise to fast-growing cell outgrowths which, 7 days after initiation of explant culture, contained a proportion of MDPCs ranging between 12-39%, based on staining for lineage-specific markers, PAX7 and MYOD. These values are comparable to those obtained from human muscle explants (10-50% Pax+ cells) (Marg et al., 2020) but lower than those reported using mouse muscle (60-90% myogenic progenitors) (Vaughan and Lamia, 2019). Moreover, I found that the proportions of myogenic cells were maintained at relatively stable levels in porcine explant-derived cultures until at least passage 2, based on sustained levels of PAX7 and MYOD (Figures 3.3 and 3.4) and their ability to robustly generate myotubes (Figure 3.8). This was followed by a gradual decrease in both PAX7 expression and myogenic potential, although MDPCs maintained their capacity to generate myotubes until at least passage 8. Loss of stemness during \textit{in vitro} culture is a well-known feature of muscle

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progenitors from several species (Fu et al., 2015, Charville et al., 2015) including pig (Ding et al., 2017). A study where porcine satellite cells were sorted based on CD56 and CD29 expression (Ding et al., 2017) reported a 30-fold decrease in PAX7 expression after 96-h in culture, with a continued more gradual decrease in both PAX7 expression and myogenic differentiation capacity during sequential passaging up to passage 10 when myotube formation became undetectable. This study achieved similar results using a much simpler, less laborious approach to obtain MDPCs. Strategies reported to extend the lifespan of myogenic precursors in culture such as addition of p38 kinase inhibitors (Ding et al., 2018) or muscle-secreted cytokines (Fu et al., 2015) to cultures may prove beneficial in extending the myogenic potential of porcine muscle explant-derived MDPCs during culture.

To my knowledge, only one previous study (Wilschut et al., 2008) had reported the use of explant culture as a source of myogenic cells in the pig; the cells obtained did not express PAX7 or MYOD and were not able to undergo myogenic differentiation unless co-cultured with C2C12 cells, although they did undergo adipogenesis and osteogenesis. The use of gelatin rather than a laminin-rich substrate such as matrigel for cell growth in that study may have accounted for their inability to obtain myogenic-rich cell populations. Matrigel has been shown to distinctly support the migration and proliferation of mice and human myoblasts while maintaining their myogenic capacity during prolonged culture in vitro (Penton et al., 2016, Wilschut et al., 2010a), and to robustly support the derivation of MDPCs from muscle explants (Wang et al., 2014, Shahini et al., 2018, Margin et al., 2020).

We used NCAM (CD56), in addition to PAX7 and MYOD, to immunophenotype explant-derived pig MDPCs. CD56 localises to satellite cells in skeletal muscle and has been widely used to isolate such cells, including in the pig (Ding et al., 2017). Of note, CD56 levels remained elevated despite a clear and dramatic reduction in the abundance of MDPCs with sequential passaging, as discussed above. Ding et al. (2017) reported a decrease in CD56 levels in porcine satellite cells during culture although to a lower extent to that detected for PAX7 or myogenic potential. Moreover, different cell types, including MSCs
(Skog et al., 2016) have been shown to express CD56 in addition to muscle stem cells, whereas recent work showed that CD56-negative cells in porcine muscle are able to generate myotubes in culture (Perruchot et al., 2021). Taken together, these data suggests that, although suitable for identifying myogenic progenitors in skeletal muscle, CD56 does not provide a specific marker of porcine MDPCs in culture.

The presence of a dynamic population of adipose progenitor cells in porcine muscle explant cultures, as indicated by 1) positive staining of MDPCs for the mesenchymal progenitor markers, PDGFRα, CD105 and CD90, and 2) the upregulation of PDGFRα and CD105 together with an increase in adipogenic capacity during extended culture, is consistent with previous data with pig muscle explants (Ceusters et al., 2017, Wilschut et al., 2008). PDGFRα is a canonical marker of fibro-adipogenic progenitors (Uezumi et al., 2014a, Uezumi et al., 2010, Uezumi et al., 2021, Joe et al., 2010a, Scott et al., 2019). Moreover, consistent with our data, studies have shown that CD105, but not CD90, marks multipotent, adipogenesis-competent precursor cell populations in human skeletal muscle (Downey et al., 2015).

These results suggest that, in contrast to the relatively rapid loss of myogenic progenitors during passaging of explant cultures, adipogenic precursors in those cultures were able to maintain their capacity for self-renewal resulting in an overall increase in adipogenic capacity of MDPCs for at least 12 passages. This is consistent with the ability of tissue derived MSCs to robustly expand and differentiate into adipocytes after extended passaging in culture (Vacanti et al., 2005, Pokrywczynska et al., 2020, Dariolli et al., 2013, Lee et al., 2015a).

There is significant evidence, both in vitro and in vivo, of a dynamic crosstalk between myogenic precursor cells and FAPs in skeletal muscle (Saccone et al., 2014, Lukjanenko et al., 2019, Wosczyna et al., 2019, Joe et al., 2010a, Uezumi et al., 2021) including inhibition of FAP adipogenesis by muscle-secreted products (Heredia et al., 2013, Kang et al., 2018, Quinn et al., 2005, Li et al., 2014) or the presence of myotubes derived from satellite cells (Uezumi et al., 2010). Those observations may to an extent explain the temporal
association observed in this study between increased adipogenic capacity and a loss in myogenic capacity of MDPCs. Moreover, the presence, as reported by others (Pisani et al., 2010a, Murakami et al., 2011, Perruchot et al., 2013a), of bipotent precursors in my explant cultures able to switch from a myogenic to an adipogenic fate in response to the changing conditions upon extended passaging cannot be discarded. In that regard, provided suitable lineage-specific antibodies for pig become available, further studies involving derivation of clonal lines coupled with lineage tracing using specific muscle and adipogenic cell markers may be undertaken in order to clarify this.

3.7 Conclusion

This study reports for the first time the robust isolation and expansion in vitro of both myogenic and adipogenic precursors from pig skeletal muscle using explant culture and provides detailed characterisation of their dynamics during extended passaging in culture. It shows that steady levels of differentiation-competent myogenic progenitors were maintained for at least two passages, and that a subsequent progressive decrease in myogenic cells was temporally associated with a gradual increase in adipogenic precursors up until at least passage 12. The study provides a new, relatively simple and convenient system to study porcine muscle dynamics in culture.
Chapter 4 Investigating the role of βKlotho (KLB) on adipogenic differentiation of porcine muscle-derived progenitor cells (MDPCs)
4.1 Introduction

4.1.1 Effects of Intrauterine Growth Restriction (IUGR) on Muscle Development

Intrauterine growth restriction (IUGR) is defined as the inability of a foetus to reach its genetically determined growth potential and is characterized by delayed or incomplete development of numerous body tissues at birth. IUGR is relatively common in pigs due to the large litter sizes typical in this species (Wu et al., 2006, Matheson et al., 2018). Among other effects, IUGR results in impaired muscle development (mostly involving reduced numbers of secondary fibres) which in turn leads to a persistent reduction in skeletal muscle growth potential (Rehfeldt and Kuhn, 2006). Reportedly the presence of fewer myofibres in IUGR pig foetuses is evident as early as day 64 of gestation, and the difference persists throughout life (Gondret et al., 2005). Similarly, differential expression of proteins involved in energy supply, protein metabolism, and muscle structure, function, and proliferation was observed in IUGR piglets indicating dysfunctional metabolism and decreased muscle growth and development (Wang et al., 2013). At slaughter, IUGR pigs produce carcasses with lower protein and higher water content, a higher proportion of fat and decreased meat tenderness which has a net detrimental impact on both the efficiency and quality of meat output (Rehfeldt and Kuhn, 2006, Gondret et al., 2005, Liu et al., 2015a).

At the cell level, muscle fibres from IUGR piglets contained fewer myogenic cell nuclei, had fewer cell yield and had a lower proportion of Ki67+ cells compared to their normal body weight litter mates (Gondret et al., 2005, Rehfeldt and Kuhn, 2006, Krueger et al., 2014, Stange et al., 2020). In addition, compared to cells from normal littermates, muscle cells from piglets with IUGR had lower viability, impaired proliferation, and reduced myogenic differentiation capacities, suggesting that IUGR has an effect on muscle progenitor cells (Stange et al., 2020, Nissen and Oksbjerg, 2009, Cortes-Araya et al., 2022).
Studies in Donadeu’s laboratory have shown that fibroblast growth factor 21 (FGF21) and its co-receptor, β-Klotho (KLB), are both elevated in IUGR foetal plasma and muscle respectively compared to their normal body littermates. Furthermore, FGF21/KLB signalling suppressed myogenesis in vitro as shown by both siRNA-mediated down-regulation of KLB and agonist activation with FGF21 in muscle progenitor cells, indicating that KLB mediates at least some of IUGR’s detrimental effects on muscle growth (Cortes-Araya et al., 2022). Also, FGF21/KLB has been shown to promote adipogenesis of muscle-derived progenitor cells in vitro (Phelps et al., 2016a, Li et al., 2020).

4.1.2 Fibroblast Growth Factor 21 (FGF21) and β-Klotho (KLB) Signalling

Fibroblast growth factors (FGFs) are a family of signalling proteins that play critical roles in a variety of biological processes, including embryonic development, postnatal growth, angiogenesis, regeneration and metabolism (Geng et al., 2020). To exert their biological actions, FGFs must bind, dimerize, and activate their cognate FGF receptor (FGFR). FGFRs belong to the family of membrane-spanning receptor tyrosine kinases. While the canonical (paracrine) FGFs are secreted proteins that function by binding (via heparin sulphate binding domain) and activating their cognate FGFRs, the endocrine FGFs (FGF19, FGF21, and FGF23) have little or no heparin sulphate binding affinity, thus they can enter the circulation and act as hormones (Kharitonenkov et al., 2005, Adams et al., 2013).

FGF19 and FGF21 are two endocrine FGFs that have been shown to have beneficial effects on lipid and carbohydrate metabolism making them potential therapeutic targets to treat obesity and related metabolic disorders (Sonoda et al., 2017). FGF21 plays critical roles in regulating energy homeostasis, lipid and glucose metabolism as well as insulin sensitivity (Geng et al., 2019). It is preferentially released in the liver in response to fasting, thus most of its metabolic actions are geared towards providing tissues with fuel as a means of overcoming nutrient deprivation (Oost et al., 2019). Other organs shown to express the FGF21 include heart (Patel et al., 2014), brown adipose tissue
In place of heparin, the transmembrane Klotho proteins, α-Klotho (KLA) and β-Klotho (KLB) have been identified as co-receptors required by endocrine FGFs to activate and dimerize their cognate receptors and activate downstream signalling pathway. While FGF19 and FGF23 require KLA, KLB is the cofactor that facilitates FGF21 activation of FGFRs thus determining the tissue specificity of FGF21 action and mediating the variety of FGF21 metabolic actions (Ding et al., 2012). KLB is a single pass transmembrane protein localized to the cell membrane and is predominantly expressed in liver, pancreas, and adipose tissue. Although FGFRs are widely distributed, it appears that liver and adipose tissue are the main FGF21 targets, which is consistent with the comparatively more restricted expression profile of KLB (Ito et al., 2000).

To exert its metabolic effects, FGF21 binds to a receptor complex comprised of an FGFR (commonly FGFR1) and the co-receptor β-Klotho (KLB). Both FGFR and KLB are present as pre-complexed proteins on the plasma membrane and are essential for effective FGF21 binding. In the absence of FGF21, the receptor complex is silent but in the presence of FGF21, KLB can bind FGF21 directly through its C-terminus and to consequently activate FGFR via the N-terminus allowing FGF21 to signal through FGFR as an activity-competent subunit (Figure 4.1) (Kharitonenkov and Larsen, 2011, Kharitonenkov et al., 2008).
Several authors have reported that FGF21 is a stress-induced hormone which primarily targets white and brown adipose tissue through the FGFR–KLB complex, and that the tissue specificity of FGF21 actions is determined by KLB expression (Dutchak et al., 2012a, Hondares et al., 2011, Muise et al., 2008, Oishi et al., 2011, Wang et al., 2008a, Chartoumpekis et al., 2011, Kharitonenkov et al., 2008). Particularly, in adipose tissue, FGF21 stimulates glucose uptake and lipolysis and also activates thermogenesis (Kharitonenkov et al., 2005, Hondares et al., 2011, Inagaki et al., 2007), actions which are lost in the absence of KLB (Ding et al., 2012, Adams et al., 2012). The reported effects of FGF21 on lipolysis have been inconsistent. On one hand, FGF21 has been shown to promote glucose transporter 1 (GLUT1)-mediated glucose uptake, lipolysis, and WAT browning in mouse 3T3-L1 cells and primary

**Figure 4.1 FGF21 receptor activation.**

(a) The FGF21 receptor complex, composed of the FGFRs and KLB that are constitutively associated on the plasma membrane, is inactive in the absence of FGF21 (b) Once FGF21 comes into the vicinity of the receptor, it binds to its receptor in a two-step process. At first, FGF21, via its C-terminus, binds to KLB. This interaction leads to a conformational change in FGFR or FGF21, or even both, allowing FGF21 through its N-terminal part to contact FGFR. Binding of FGF21 to FGFR/KLB complex triggers the intrinsic tyrosine kinase activity of FGFR, followed by receptor cross phosphorylation, downstream signal transduction and a cellular functional response. Adapted from (Kharitonenkov and Larsen, 2011).
human adipocytes (Inagaki et al., 2007, Kharitonenkov et al., 2005). Other studies, however, have found that FGF21 reduces lipolysis in primary cultures of human and mouse adipocytes, as well as mouse 3T3-L1 cells (Badman et al., 2009, Li et al., 2009, Arner et al., 2008, Fisher et al., 2012).

In skeletal muscle, FGF21 is released in response to different kinds of stress including starvation, mitochondrial myopathies, endoplasmic reticulum stress, obesity, and aging (Keipert et al., 2014, Pereira et al., 2017). According to Oost et al. (2019), the FGF21 that is released from skeletal muscle in response to fasting reduces protein synthesis and boosts autophagy, which reduces the overall mass of muscles. Also, loss of muscle mass has also been linked to FGF21, which is generated in muscle in response to mitochondrial malfunction (Tezze et al., 2017).

There are limited studies on how KLB affects skeletal muscle and adipose tissue, however, based on their expression patterns and known molecular functions, both KLA and KLB have been linked to the regulation of adiposity (Ito et al., 2000, Mizuno et al., 2001, Chiara et al., 2006). KLB upregulation in fibro/adipogenic progenitors (FAPs) derived from mouse skeletal muscle has been linked to increased adipogenic differentiation (Phelps et al., 2016a). Similarly, FGF21 has been shown to increase adipogenesis while simultaneously reducing osteogenesis in bone marrow-derived mesenchymal cells from Dystrophin/utrophin double-knockout mice. Interestingly, these effects were ameliorated by siRNA mediated downregulation of KLB (Li et al., 2020). Furthermore, increased expression of KLB in skeletal muscle has been associated with muscle pathology exhibiting enhanced fibrosis and adipogenesis (Stuilsatz et al., 2012a).

4.1.3 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated proteins (Cas) System

Genetically modified animal and cell models are extremely useful for understanding gene function in growth, development, and disease. Since its
discovery, the Clustered regularly interspaced palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) (CRISPR/Cas9) system has undergone significant improvements and is now widely used for genome editing in both basic biological research and biomedicine, thereby revolutionising the field of molecular biology.

CRISPR/Cas9 system is an adaptive immune system used by archaea and bacteria to recognize and rapidly respond to invading pathogens such as bacteriophage or plasmid (Barrangou et al., 2007). Cas9 is a nuclease guided by small RNAs through Watson-Crick base pairing with target DNA.

Although three distinct CRISPR systems (I, II, and III) have been discovered, the Type II CRISPR system which was adapted from that used by Streptococcus pyogenes is currently the most popular. It consists of Cas9 endonuclease, the target-specific CRISPR RNA (crRNA) array encoding a short guide RNA sequence (20 nucleotide long) and a target-independent trans-activating crRNA (tracrRNA) (Jinek et al., 2012a, Cho et al., 2013, Garneau et al., 2010). The 20-nucleotide guide sequence can be designed to target a specific sequence complementary to the target DNA. To induce DNA double strand breaks (DSB), the tracrRNA hybridizes with the crRNA to form an RNA duplex structure which acts as a guide for the Cas9 nuclease attracting it to the cleaving complex.

As a prerequisite, Streptococcus pyogenes Cas9 (SpCas9) requires the target DNA to immediately precede a conserved NGG sequence (where N can be any base) that is 3’ downstream of the protospacer sequence referred to as the protospacer adjacent motif (PAM) (Sternberg et al., 2014, Chylinski et al., 2013, Jinek et al., 2012b). The Cas9: tracrRNA:crRNA cleaving complex (Cas9:gRNA complex) scans the target DNA for the PAM which allows the gRNA to bind complementary to the target DNA thereby activating the nuclease activity of Cas9. Once bound, two distinct domains of the SpCas9 viz; the HNH nuclease domain and RuvC domain at the amino terminus nicks the sense and antisense strand respectively inducing a blunt DSB 3 base pairs
proximal to the PAM (Figure 4.2) (Nishimasu et al., 2015, Sapraauskas et al., 2011, Jinek et al., 2014, Chylinski et al., 2013).

There are two dominant endogenous repair mechanisms employed for the repair of DNA DSB; non-homologous end joining (NHEJ) or homology-directed repair (HDR). The difference between these mechanisms is in the requirement of a repair template and the initial processing of the DNA ends (Lino et al., 2018). NHEJ is the primary cellular DNA repair mechanism and involves the protein-mediated re-ligation of the broken DNA strand consequently introducing small nucleotide insertions/deletions (indels) at the cut site (Hefferin and Tomkinson, 2005). NHEJ is considered an error-prone repair mechanism because it does not require a homologous DNA template to direct repair. HDR on the other hand results in an error-free repair of DSB as it requires homologous repair template which can be a single-strand DNA (ssDNA) oligonucleotide or a dsDNA plasmid to precisely repair the DSB (Wang et al., 2008b, Takata et al., 1998). HDR is commonly utilised when there is a need to introduce exogenous DNA templates at the site of DSB.

If the DSB occurs in the coding sequence of a gene, it would result in a range of possible editing outcomes ranging from gene insertion or deletion, amino acid modifications to frame shifts and functional gene knockout. NHEJ generally introduces indels resulting in frameshift mutation causing a premature termination of protein translation, nonsense-mediated decay of the mRNA transcript and protein loss (Ran et al., 2013, Cong and Zhang, 2015). On the other hand, HDR uses DNA template during repair resulting in gene correction or gene addition thereby making specific changes to a gene (Whitelaw et al., 2016, Lino et al., 2018).
Figure 4.2 Type II CRISPR/Cas9 system.

(A) Schematic illustration of the Type II CRISPR/Cas9 system showing the single-guide RNA (sgRNA) made up of a 20-nt guide sequence (blue) directly upstream of the protospacer adjacent motif (PAM), NGG (orange circles) and a scaffold (red) that targets the Streptococcus pyogenes Cas9 nuclease (green) to genomic DNA to generate a double-strand DNA break (DSB) 3 bp upstream of the PAM (red triangles). (B) Schematic representation showing the two types of DSB repair mechanisms; non-homologous end-joining (NHEJ) which creates random insertions or deletions at the target site or homology-directed repair (HDR). For HDR, two types of templates can be used: a small single-stranded DNA (ssDNA) oligonucleotide donor with short homology arms of 60-70 bp and a linear or circular dsDNA plasmid with long homology arms of 1 to 3 kb. Adapted From (Doetschman and Georgieva, 2017).

Following its first use as a genome editing tool in mammalian cell cultures (Jinek et al., 2013, Mali et al., 2013), the CRISPR/Cas9 has been widely used as a genome editing tool in muscle progenitors, most notably to experimentally correct dystrophin mutations in human Duchenne muscular dystrophy (DMD) cells or mdx mice (a mouse model of DMD). One study showed that the dystrophin mutation in muscle stem cells obtained from mdx mice could be corrected using CRISPR/Cas9-mediated genome editing, and that dystrophin
expression was restored when the cells were transplanted into mdx mice (Zhu et al., 2017). Also, Tasca et al. (2022) achieved a restoration of full-length dystrophin synthesis in DMD myoblasts using adenovirus delivery of donors in conjunction with matched CRISPR/Cas9 components. Furthermore, several studies have successfully restored truncated dystrophin protein expression in primary mdx mice or DMD myoblasts via CRISPR-induced deletion or CRISPR-mediated excision of mutated exon 23 of the DMD gene or other exons, resulting in improved muscle biochemistry and function in mdx mice. (Xu et al., 2016, Long et al., 2016, Iyombe-Engembe et al., 2016, Nelson et al., 2016, Tabebordbar et al., 2016).

There are limited studies on the applicability and efficiency of CRISPR/Cas9 system in the pig. In one study, somatic gene editing using CRISPR/Cas9 reportedly induced the expression of a shortened dystrophin and improved skeletal muscle function in a pig model of DMD lacking exon 52 (Moretti et al., 2020). Particularly, in vitro studies describing the use of CRISPR/Cas9 system in porcine embryonic and foetal fibroblast have been reported by several groups (Su et al., 2018, Sato et al., 2014, Li et al., 2017b, Gerlach et al., 2018) but its applicability in pig primary muscle stem cells is yet to be reported.

4.1.4 Experimental Aims;

The major aim of the studies described in this chapter was to use the muscle development model described in chapter 3 to investigate the effects of KLB/FGF21 signaling in adipogenesis of porcine MDPCs using both a siRNA mediated knockdown and CRISPR/Cas9-mediated knockout of KLB.

Specific objectives were;

1. To investigate the effects of increasing concentrations of FGF21 on the ability of porcine MDPCs to undergo adipogenic differentiation in vitro.

2. To investigate the effects of KLB on adipogenic differentiation of pig MDPCs in vitro using siRNA- mediated KLB down-regulation.
3. To establish a protocol for CRISPR/Cas9 editing of porcine MDPCs and investigate the effects of CRISPR/Cas9-mediated $\textit{KLB}$ deletion on adipogenic differentiation of pig MDPCs.
4.2 Materials and Methods

4.2.1 Porcine MDPCs Isolation and Culturing

Porcine MDPCs were isolated from 4-week-old piglet and cultured as detailed in Chapter 3, Section 3.4.1. For single-cell clonal culture, cells were FAC sorted into gelatine coated 96 well plates containing 50 µl of conditioned media (prepared by mixing 40% conditioned growth media and 60% growth media). After 48 hrs, 100 µl was added to each well. After 1 week, 100 µl was gently removed and replaced with 120 µl of fresh conditioned media. When cells reached 40-50% confluency, the cells were transferred to 48 well plates. At 50% confluency, one 48 well was split into 2, and 1 well was used for genotype analysis while the other well was used for cell expansion.

4.2.2 FGF21 Stimulation of MDPCs

Pig MDPCs seeded on collagen were cultured in standard growth media till they were confluent. On the day of differentiation, the media was changed to adipose induction media (DMEM + 10% FBS, 1% P/S, 0.5 mM IBMX, 1 µM dexamethasone, 100 µM indomethacin and 10 µg/ml insulin) which was supplemented with 1, 10 or 100 ng/ml FGF21 (h-FGF21, PeproTech cat #100-42). Similarly, adipose maintenance media (DMEM + 10% FBS, 1% P/S, and 10µg/ml insulin) used from day 4 of differentiation was also supplemented with the respective doses of FGF21. Control wells contained only adipose induction media or adipose maintenance media. Samples were collected on day 14.

4.2.3 MDPCs Transfection with Small Interfering RNA (siRNA)

The siRNA sequences used were pre-designed and obtained from Dharmaco (Horizon Discovery Ltd). The sequences were siKLB 1- GAACCAACAGAUCAGAAAUU and siKLB 2- CGUUGGAAACUGGAGCAUUUUU and Negative siRNA control (Qiagen, cat # 1022076). siRNA or control was transfected into pig MPDCs using the
HiPerfect transfection reagent (Qiagen, cat # 301704). First, cells were seeded in 24 well plates and cultured in standard growth media till they were 60% confluent. On the day of transfection, 25 nM each of siKLB 1 and siKLB 2 or 50 nM of negative siRNA control was diluted in culture media and HiPerfect reagent was added (3μl/ 100μl transfection volume). The siRNA/ HiPerfect transfection mix was incubated for 10 min at room temperature. The cells were washed once in DMEM, and 250 μl of fresh media was added. Next, 100 μl of the siRNA-transfection-mix or control-transfection-mix was added to the respective cells dropwise and given a gentle swirl to mix before transferring the plates to the incubator set at 39°C and 5% CO2. After 6-8 hours, 150 μl of standard growth media was added to each well.

At 24 hours post-transfection, the culture media was changed to adipose induction media (DMEM + 10% FBS, 1% P/S, 0.5 mM IBMX, 1μM dexamethasone, 100 μM indomethacin and 10 μg/ml insulin) and differentiated as described in section 3.4.7.

### 4.2.4 MDPCs Transfection with DNA

Pig MDPCs were transfected with plasmid DNA using either the Neon™ Transfection system (Invitrogen; cat# MPK5000, MPK10025, and MPK 1025) or Lipofectamine™ 3000 (Invitrogen; cat# L3000001) following manufacturer's instructions.

#### 4.2.4.1 Neon™ Transfection

DNA was transfected in MDPCs using a Neon™ electroporation system with 10 μl or 100 μl reaction tips. Two days before transfection, MDPCs frozen in 5% DMSO were quickly thawed and seeded in a T75 flask with an anticipation to reach 60-70% confluency. On the day of transfection, cells were washed in PBS, digested with Trypsin-EDTA (Gibco, cat # 25200072) and inactivated in serum-containing media. The cell suspension was centrifuged at 200 x g for 5 min at room temperature, supernatant removed, and cell number determined. Next the cells were resuspended by gently pipetting up and down in resuspension Buffer (R) at a density of 5x10^6 cells/ml.
Next 0.5 µg or 1 µg of DNA was added to each tube, mixed by gently flicking the tube and taken up by an appropriately sized tip attached property to a neon transfection pipette. The pipette and tip were then inserted into a cuvette containing 3 ml of electrolyte buffer (buffer E for 10 µl tip or buffer E2 for 100 µl tip) inserted in the Neon™ pipette station. The optimized condition of 1650 v pulse voltage, 10 ms pulse width and three electrical pulses was applied, and the cuvette was monitored for sparks. Once the process was completed, the pipette tip was detached from the device and the cells were then transferred to gelatine-coated 6-well plate or 24- well plate containing standard growth media without antibiotics. The plates were then incubated at 39°C and 5% CO2 for 24 or 48 hours.

### 4.2.4.2 Lipofectamine™ 3000 Transfection

24 hours before transfection, the porcine MDPCs were seeded at a density of 0.2 x 10⁶ cells per well of a 6-well plate to aimed to achieving 70% confluency on the day of transfection. On the day of transfection, cells were prepared for transfection by gently washing with pre-warmed DPBS to remove serum. The media was replaced with 2 ml of Opti-MEM I Reduced Serum Medium (Opti-MEM I; Gibco cat# 11058-021) and incubated for 1 hour at 39°C.

The transfection mixture for a single well was prepared as follows; 3.75 µl of Lipofectamine™ 3000 (Invitrogen, cat # L3000001) was diluted in 125 µl of Opti-MEM I in a tube. In a second tube 2 µg or 5 µg of plasmid DNA and 4 µl or 10 µl of Lipofectamine™ 3000 reagent respectively was added to 125 µl of Opti-MEM I. Next, the 125 µl Lipofectamine/Opti-MEM mixture was gently mixed with the DNA/Opti-MEM mix to obtain the DNA complex which was then incubated for 20 min at room temperature. Finally, the DNA complex was added dropwise on the cells and incubated at 39°C and 5% CO2 for 24 hours. The Opti-MEM media was replaced with standard growth media after 24 hours and incubated for another 24 hours.

In both transfections, at 24hrs and 48hrs post-transfection, cells were visualized using a Nikon Eclipse TE2000-U inverted microscope fitted with a digital camera with a scion software programme.
4.2.5 Complementary DNA (cDNA) Synthesis and Quantitative Real-time PCR (qPCR) Analysis

Samples were collected on specific days in Trizol and RNA was extracted as described in section 2.5.7. cDNA was synthesized from RNA (0.5 µg) using Superscript III reverse transcriptase kit (Invitrogen, cat #18080-044). Genes of interest were analysed by qRT-PCR using Sensi-FAST SYBR Lo-ROX kit (Bioline UK, cat #BIO-94050) in a Stratagene MX 3005P machine and data analysed with MxPro Software. The detailed protocol is in section 2.5.9. Gene expression was measured relative to a standard curve prepared from a pool of samples being tested and normalized against two reference genes (18s and RPL4). The primers used are listed in tables 2.1 and 4.1.

4.2.6 Immunofluorescence Analysis of MDPCs

Immunostaining of porcine MPDCS for KLB was done as described in section 2.5.10. Cells were stained with Anti-KLB antibody (1:50, Abcam, cat #106794) overnight and secondary anti-rabbit IgG conjugated to AF568 (1:500, Invitrogen, cat# A10042). Images were captured on a Zeiss Axiovert 25 inverted microscope fitted with Zen software and analysed using ImageJ (Fiji) software.

4.2.7 Fluorescence-activated Cell Sorting

Transfected cells were dissociated after 24 hours, resuspended in 300 µl of media (5%FBS in Ham’s F-10 nutrient mix, (Gibco, cat# 11550043), and filtered through 35 µm nylon mesh (BD Biosciences: cat# 352235) into a sterile tube to remove cell clumps. FACS analysis was done using BD FACSaria III™ sorter (BD Biosciences, San Jose, CA, USA) and cells were either sorted individually into a single well of a 96 well plate containing 40% conditioned medium and 60% culture medium or collected as a mixed cell pool.
4.2.8 Plasmid DNA Isolation

4.2.8.1 Small Scale Plasmid DNA Isolation (Mini-prep)

For the preparation of plasmid on a small scale, plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen, Cat #: 27104) according to the manufacturer’s instructions. All centrifugation was performed at 13,000 rpm in a table-top centrifuge unless otherwise stated.

For each plasmid needed, a single bacterial colony was picked from a plate with a sterile pipette and inoculated in 5 ml of Luria- Bertani (LB) media containing 100 μg/ml ampicillin (Sigma Aldrich, Cat# A0166). The LB media was then cultured in an orbital shaking incubator at 37°C with shaking at 220 rpm overnight. 3 ml of overnight bacterial culture was centrifuged at 6800 x g for 5 min to pellet the bacteria. The bacterial pellet was then resuspended completely in 250 µl of buffer P1 containing RNASE A followed by the addition of 250 µl of buffer P2. The resulting solution was mixed thoroughly by inverting the tube a few times and incubated for 5 min at room temperature to lyse the bacteria.

After 5 min, 350 µl of buffer N3 was added to the solution and mixed immediately to neutralize the lysis reaction followed by centrifuging the tubes for 10 min to pellet cell debris. The supernatant was transferred to a QIAprep 2.0 Spin Column, incubated for 1 min and subsequently centrifuged for 1 min to allow the plasmid DNA bind to the column. The bound DNA was washed once with 500 µl of buffer PB, centrifuged for 1 min and the flow-through discarded. The column was washed one more time with 750 µl of Puffer PB, centrifuged for 1 min and flow-through discarded. The column was centrifuged for an additional 1 min to remove residual wash buffer from the matrix. Finally, to elute bound DNA, the spin column was transferred to clean 1.5 ml Eppendorf, 50 µl of buffer EB added to the centre of the column membrane, incubated at room temperature for 1 min and then subsequently centrifuged for 1 min.
The plasmid DNA concentration was quantified using a Nanodrop spectrophotometer (NanoDropTM 1000, Thermo Scientific) and stored at -20°C until required.

4.2.8.2 Large Scale Plasmid DNA Isolation (Maxi-prep)

It is important to use endotoxin-free plasmid DNA for cell transfections, therefore, large-scale plasmid DNA was isolated using Endotoxin-Free (EndoFree) Plasmid kit (Cat#12362, Qiagen) following the manufacturer's instructions.

Briefly, 2 ml of overnight bacterial culture was transferred into 100 ml LB medium containing 100 μg/ml ampicillin and cultured at 37°C in an orbital shaking incubator set at 220 rpm overnight. After 16 hours, the bacterial culture was centrifuged at 4,000 x g at 4°C for 20 min to pellet the bacteria. The resulting supernatant was discarded, and the pellet was resuspended in 10 ml of buffer P1 containing RNAse A and LyseBlue reagent. Subsequently, 10 ml of buffer P2 was added to the resuspended pellet, mixed by inverting the tube, a few times and incubated for 5 min to lyse the bacteria.

After 5 min, 10 ml of chilled buffer P3 was added to the lysate to neutralize the lysis buffer and mixed by inverting the tubes a few times until the lysate became less viscous. The lysate was then transferred to a QIAfilter cartridge, incubated for 10 min, and allowed to filter through into a clean tube. Next, 2.5 ml of buffer ER was added to the filtered lysate, mixed by inverting the tube several times and incubated for 30 min on ice to prevent binding of endotoxins. While the lysate was incubating on ice, the QIAGEN-tip was equilibrated by adding 10 ml of buffer QBT and allowed to empty by gravity flow.

After 30 min, the lysate was transferred to the column, allowed to flow through and enable the plasmid DNA bind to the column. Subsequently, the QIAGEN-tip was washed twice with 30 ml of buffer QC and DNA eluted with 15 ml buffer QN into a clean tube. Subsequently, 10.5 ml of isopropanol was added to the DNA to precipitate the DNA and centrifuged at 4,000 x g for 60 min at 4°C to pellet the precipitated DNA. The DNA pellet was then washed in 5 ml of 70%
ethanol, centrifuged at 4,000 x g for 30 min at 4°C, air dried to remove ethanol and dissolved in 200 μl of buffer TE.

The plasmid DNA concentration was quantified using a Nanodrop spectrophotometer (NanoDrop™ 1000, Thermo Scientific) and stored at -20°C until required.

4.2.9 Genomic DNA Extraction

4.2.9.1 Genomic DNA Extraction with Qiagen kit

Genomic DNA (gDNA) was extracted from cultured cells using DNeasy Blood & Tissue Kit (Qiagen, Cat# 69504) following the manufacturer’s instructions. All centrifugation was performed at 6,000 x g in a table-top centrifuge at room temperature unless otherwise stated.

Briefly, 0.2-0.5 x10⁶ cells were centrifuged at 300 x g for 5 min to pellet the cells. The cell pellet was resuspended in a solution containing 200 μl PBS, 20 μl proteinase K and 20 μl RNASE and incubated at room temperature for 2 min to disrupt the pellet. Next, 200 μl buffer ATL was added to the reaction, mixed thoroughly by vortexing for 2 sec and incubated for 10 min at 56°C in a water bath. Then, 200 μl ethanol (96–100%) was added to the sample and mixed before transferring to a DNA Mini spin column which was centrifuged for 1 min. The column was inserted into a new collection tube, 500 μl Buffer AW1 was added and centrifuged for 1 min to wash the column. The column was washed a second time with 500 μl Buffer AW1 and centrifuged at 16,000 x g for 3 min. Finally, the spin column was transferred to a clean 1.5 ml Eppendorf tube, 100 μl of water was added to the membrane and incubated for 1 min followed by centrifuging at 12,000 x g for 1 min to elute the DNA.

The DNA concentration was quantified using a Nanodrop spectrophotometer (NanoDrop™ 1000, Thermo Scientific) or the Qubit® Fluorometer (Qubit™ 4 Fluorometer, Invitrogen) using Qubit® dsDNA BR Assay Kits (Invitrogen, cat # Q32850) and stored at 4°C until required.
4.2.9.2 Genomic DNA Extraction with Quick Extract (QE)

For a small number of cells and single cell clones, gDNA was isolated using the QuickExtract™ extraction solution (Cambio Cat #QE09050). Briefly, cells were washed once in PBS before adding 50 μl or 100 μl per 96 or 24 well plates respectively to lyse the cells. Lysed cells were harvested by pipetting several times and transferred to 0.2 ml Eppendorf tubes. The samples were incubated for 15 min at 65°C, 15 min at 68°C and 10 min at 98°C in a thermocycling PCR machine. The gDNA was used immediately for PCR amplifications (Section 4.2.10) or stored at -20°C until required.

4.2.10 PCR Primers and DNA Amplification with Polymerase Chain Reactions (PCR)

PCR primers used in this chapter were designed using "Geneious Prime 2020.0.3 (https://www.geneious.com)" or NCBI blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers were synthesized by Invitrogen or Fisher scientific and diluted to 100 μM (stock concentration) in nuclease-free water and kept at -20°C for long-term storage. All primers are listed in table 4.1.

PCR was performed with two different kits as described below.

4.2.10.1 Routine PCR using Taq DNA Polymerase

Using 100 ng of DNA, PCR was performed in a total volume of 50 μl reaction per sample with Taq DNA polymerase (Invitrogen: Cat# 10342020) following the manufacturer’s instructions. For each reaction, the components include, template DNA (volume corresponding to 100 ng), 1 μl dNTP mix (10 mM), 1 μl each of 10 μM forward and reverse primers, 5 μl 10X Taq Buffer, 0.5 μl Taq DNA polymerase, and Nuclease free water added to make a final volume of 50uL.
The reagent concentration used and the thermal cycling profile are shown in table 4.2.

**Table 4.1 List of PCR and sequencing primers used in this thesis.**

SD_KLB P1 and SD_KLB P2 were used to amplify the target regions to verify guide sequences; SD_KLB P4 and SD_KLB P8 were used to amplify regions outside the guides to verify deletion; SDJ-KLBa-E5 For, and SDJ-KLBa-E5 Rev were used for RT-qPCR to amplify exon 5.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Direction</th>
<th>Sequence 5' - 3'</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD_KLB P1</td>
<td>For</td>
<td>ATGATCTCCAGGGAATGAATGGA</td>
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<td>SD_KLB P2</td>
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<td>For</td>
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<td>SDJ-KLBa-E5</td>
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**Sequencing primers**

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Direction</th>
<th>Sequence 5' - 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD_KLB S1</td>
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<td>TCTTCAGCACCAGATGAAAGA</td>
</tr>
<tr>
<td>SD_KLB S2</td>
<td>For</td>
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</tr>
<tr>
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<td>For</td>
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</tr>
<tr>
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<td>For</td>
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</tr>
<tr>
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<td>For</td>
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<tr>
<td>oSL_35</td>
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<td>GTCAATAGGGGGCGTACTTG</td>
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</table>

For- Forward Primer. Rev- Reverse Primer
Table 4.2 PCR Conditions with Taq DNA Polymerase

Samples were prepared on ice following the sequence on the table. “S” is the annealing temperature calculated using the online NEB annealing temperature calculator (https://tmcalculator.neb.com/). For each run, a negative control containing no DNA template was included to check for reagent contamination.

<table>
<thead>
<tr>
<th>Components</th>
<th>Final Concentration</th>
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<tr>
<td>DNA template</td>
<td>100ng</td>
</tr>
<tr>
<td>Forward primer (10 μM)</td>
<td>200 mM</td>
</tr>
<tr>
<td>Reverse primer (10 μM)</td>
<td>200 mM</td>
</tr>
<tr>
<td>dNTP mix (10 mM)</td>
<td>0.2 mM</td>
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<tr>
<td>5X HF reaction buffer</td>
<td>1X</td>
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<td>Nuclease free water</td>
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<tr>
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<td>Extension</td>
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<td>Final extension</td>
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**4.2.10.2 Routine PCR using Phusion® High-Fidelity DNA Polymerase**

PCR was performed with 50-100 ng of DNA in a final volume of 25 μl using the Phusion® High-Fidelity DNA Polymerase kit (ThermoFisher Scientific: Cat# F-553S) according to the manufacturer’s instruction. For each PCR, the components were 0.25 μl Phusion high-fidelity polymerase, 0.50 μl dNTP mix
(10 mM), 1.25 µl of 10 µM Forward primer and reverse primers, 5 µl 5X HF reaction buffer, 11.75 µl Nuclease free water and 5 µl DNA sample (10-20 ng/µl). The final concentration of reagents used, and the thermal cycling profile is shown in Table 4.3

Table 4.3 PCR Reaction conditions with Phusion® High-Fidelity DNA Polymerase

Samples were prepared on ice. For each run, a negative control containing no DNA template was included to check for reagent contamination. “S” is the annealing temperature calculated using the online NEB annealing temperature calculator (https://tmcalculator.neb.com/).

<table>
<thead>
<tr>
<th>Components</th>
<th>Final concentration</th>
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<td>DNA template</td>
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<td>Forward primer (10 µM)</td>
<td>250 mM</td>
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<tr>
<td>Reverse primer (10 µM)</td>
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<tr>
<td>dNTP mix (10 mM)</td>
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</tr>
<tr>
<td>5X HF reaction buffer</td>
<td>1X</td>
</tr>
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<td>Nuclease free water</td>
<td>Up to 25 µl</td>
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<td>Phusion high-fidelity polymerase (2 U/µl)</td>
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<th>Thermocycler conditions</th>
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<tr>
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4.2.11 DNA Visualization with Agarose Gel Electrophoresis

To prepare 1% agarose gels used, 1.6 g of UltraPure™ Agarose (Invitrogen: #16500500) was dissolved in 160 ml of TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA; supplied by Central Services Unit, Roslin Institute) and then heated in a microwave for 2 min to dissolve the agarose. When the solution was cool to touch, 10% total volume of GelRed® nucleic acid gel stain (Biotium: Cat #41003) was added to the agarose solution, mixed by swirling, and poured into a casting tank. To create the wells, a gel comb was inserted into the agarose solution in the casting tank and then allowed to set in the dark. Each PCR product was mixed to a final concentration of 1x with gel loading dye purple (6X), no SDS (NEB, Cat# B7025S), and loaded into each well of the gel which was ran at 80-100 volts for 40-60min until markers were distinguished. For each run, NEB 1 kb Plus DNA ladder (NEB, Cat# N0559S) was loaded as a size marker. Subsequently, gels were visualized with the ultraviolet Transilluminator and captured with the NuGenius gel documentation system (Syngene, Cambridge UK).

4.2.12 Purification of DNA from Agarose Gels

PCR products were excised from agarose gels and the DNA was extracted using QIAquick Gel Extraction Kit (Qiagen, Cat# 28704) following the manufacturer’s instructions. All centrifugation steps are carried out at 12,000 x g (13,000 rpm) in a conventional table-top microcentrifuge at room temperature (15–25°C).

Briefly, gel slices were weighed and three volumes of buffer QG were added per gel weight (100 mg equals 100 μl). The samples were heated at 50°C in water for 10 minutes with intermittent vortexing until the gel was completely dissolved. Then 1 volume of isopropanol was added to the sample to increase DNA yield, mixed, and transferred to a QIAquick spin column inserted in a 2 ml collection tube and centrifuged for 1 min to allow the DNA bind to the column. Next, 500 μl Buffer QG was added to the column and centrifuged for
1 min to remove any traces of agarose. To wash, 750 μl Buffer PE was added to the column and centrifuged for 1 min. The column was centrifuged for another 1 minute to remove residual ethanol. Finally, the column was transferred to a clean Eppendorf (1.5 ml), 50 μl of water was added to the centre of the column and incubated for 1 min followed by a 1 min centrifugation step to elute the DNA.

The DNA was quantified with the Nanodrop spectrophotometer and stored at -20°C

4.2.13 DNA sequencing

Sanger sequencing was performed by DNA Sequencing Facility, Institute of Genetics and Cancer, University of Edinburgh or DNA Sequencing and Services, MRC/PPU, College of Life Sciences, the University of Dundee, Dundee, Scotland. Plasmid DNA sequencing was done with 10 μl of 100-150 ng/ μl DNA and 2.5 μl of 5 μM primer. Before sequencing, PCR products were purified using MinElute PCR Purification Kit (Qiagen: Cat #28and 004), eluted in 50 μl of nuclease free water. For sequencing, 30 μl of purified PCR products and 100 μM of the appropriate primer was used.

All sequencing data was obtained as ABI files and analyzed using Geneious Prime 2020.0.3 software and Lasergene (DNASTAR series).

4.2.14 Selection of gRNA and Assembly of CRISPR/Cas9 Vectors

To select suitable gRNAs, the target genomic DNA sequence was imputed into the web tool, CHOPCHOP (http://chopchop.cbu.uib.no/) (Labun et al., 2019) specifying the specie the reference belongs to, the site-specific nuclease to be used as well as the purpose (whether knock out, knock in etc.). The output was a ranking of suitable gRNA sequences with computational predictions of on- and off-target scores for each intended guide. The gRNA oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (IDT) and the sequence details are shown in table 4.4. Please note that for efficient
transcription of human U6 RNA polymerase III, the 5' of the gRNAs should be a 'Guanine (G)' nucleotide.

The CRISPR plasmids used in this thesis were the PX458 (pSpCas9(BB)-2A-GFP), a gift from Feng Zhang (Addgene plasmid # 48138; http://n2t.net/addgene:48138; RRID:Addgene_48138) (Ran et al., 2013) and the PX458-mcherry which was a gift from Joanna Wysocka (Addgene plasmid # 161974; http://n2t.net/addgene:161974; RRID:Addgene_161974) (Gu et al., 2020). Both PX458 plasmids has a BbsI restriction enzyme site that allows insertion of gRNA oligo inserts for co-expression of gRNA and Cas9 protein and a hU6 promoter driving the transcription of the gRNA. The gRNA oligos were inserted into PX458 vector following a published protocol described in (Ran et al., 2013). The reaction was transformed into XL10-Gold® ultracompetent bacterial cells and correct insertions confirmed by PCR and Sanger sequencing.

Table 4.4 List of gRNAs used.

<table>
<thead>
<tr>
<th>gRNA Sequences</th>
<th>For Oligo 5'</th>
<th>3'</th>
<th>Rev Oligo 5'</th>
<th>3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLB-gRNA1</td>
<td>caccAGATCTTTGCAGTCCCCCGC</td>
<td>aaacGCGGGGGACTGCAAAGATCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KLB-gRNA2</td>
<td>caccAGAATCCAGTAACAGCTCGT</td>
<td>aaacACGAGCTGTTACTGGATTCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KLB-gRNA3</td>
<td>caccGTAGGAGAATAAAGCTGAC</td>
<td>aaacGTCAGCGTTTATTCCTCCAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KLB-gRNA4</td>
<td>caccGTGGAAAAAGGCCCTCTATAT</td>
<td>aaacATATAGGGGTCTTTTCCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KLB-gRNA5</td>
<td>caccGATGAAAATAATCCCATATAG</td>
<td>aaacCTATATGGGATTTTTTCATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KLB-gRNA6</td>
<td>caccgAACTGGAGCATTTCAGGTTGG</td>
<td>aaacCCTCACCCTGAAATGCTCCA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

‘cacc’ (red) is the 5' overhangs compatible with BbsI restriction enzyme sites representing the terminal sequence of the U6 promoter in PX458 and 20-bp gRNA sequences in bold letters.
4.2.15 ICE and TIDE Analysis

The web-based tools, ‘Inference of CRISPR Editing (ICE)’ (https://ice.synthego.com) (Conant et al., 2022) and ‘Tracking of Indels by DEcomposition (TIDE)’ (https://tide.nki.nl) (Brinkman et al., 2014a) were used to assess gRNA cleavage efficiency. PCR amplification products spanning the target region were subjected to Sanger sequencing. The resulting sequence trace files of control and an edited sample as well as the gRNA sequence were loaded into the TIDE or ICE web tool. In both cases, the gRNA sequence was used as a reference to determine the expected cut site and by comparing trace files of control and edited samples, the cutting efficiency, frequencies of indels, and indel spectrum were calculated.

4.2.16 Statistical analysis

Minitab 20 statistical software was used for all statistical analysis and Graphs were produced with GraphPad PRISM software version 9.0.2. Kolmogorov–Smirnoff test was used to assess normal distribution of the data and log-transformed transformed before analysis if required. Statistical significance was determined using one-way or two-way ANOVA unless otherwise stated. Tukey test was used to compare among means and statistical significance was set at P<0.05.
4.3 Results

4.3.1 Activation of KLB by Stimulation with Fibroblast Growth Factor 21 (FGF21) Enhances Adipogenic Differentiation of Porcine MDPCs.

To determine the effects of stimulation of MDPCs with FGF21, MDPCs were seeded on collagen and cultured in adipogenic media supplemented with different doses of FGF21. Adipogenic differentiation increased progressively with increasing levels of FGF21 as confirmed by an increase in FABP4 transcript levels together with a tendency for higher PPARγ levels in cells treated with the highest dose of FGF21 (Figure 4.3A-C).

Figure 4.3 Effects of FGF21 stimulation on adipogenic differentiation of porcine MDPCs

(A) Representative Oil red O-stained images of MDPCs differentiated for 14 days in the presence of different concentrations of FGF21 as indicated. Scale bar =50 µm. (B) mRNA levels, measured by RT-qPCR of (B) FABP4 and (C) PPARγ transcripts in MDPCs differentiated for 14 days in the presence of various concentrations of FGF21 as indicated. Data are shown as Mean ± SEM from three independent experiments. Means with different superscripts (a, b) are different (P<0.05). n=3 biological replicates. P values shown on graph.
4.3.2 RNAi-mediated Downregulation of KLB Inhibits Adipogenic Differentiation by Porcine MDPCs

To assess if KLB plays a role in adipocyte formation in pig MDPCs, I tested the effect of downregulating KLB in MDPCs during adipogenic differentiation. MDPCs were seeded on collagen and cultured as described in section 2.5.6. At 70% cell confluency, they were transfected with siKLB or a scrambled sequence (control cells) and the media was changed to adipose induction media 24 hours later. As the effects of siRNA on expression of target genes are transient, cells were re-transfected after 72 hours to maintain KLB at reduced levels for the remaining of the experiment.

In control cells, KLB levels were initially undetectable (Days 0 and 1) and increased progressively thereafter. In contrast, transfection with siKLB prevented an increase in KLB during the 7-day experiment (Figure 4.4A). This was confirmed by reduced protein expression on day 2 and day 4 of differentiation in siKLB treated MDPCs compared to control cells, as revealed by immunocytochemistry analyses (Figure 4.4B). As controls, unstained cells, an IGG negative control, and a secondary control (antibody alone) were used. Taken together, this result indicates that RNAi was highly efficient in preventing KLB upregulation during adipogenic differentiation of porcine MDPCs.

Moreover, KLB downregulation significantly reduced the ability of pig MDPCs to form mature adipocytes as shown by oil red O staining of intracellular lipids (Figure 4.5A). This was confirmed by the reduced expression of genes associated with adipogenesis; the transcription factor, *PPAR*γ, and a fatty acid carrier protein, *FABP*4, in siRNA-transfected cells (Figure 4.5B).
Figure 4.4 siRNA transfection and KLB expression during the differentiation of MDPCs.

(A) Relative expression of KLB transcript in siKLB- or scrambled siRNA- transfected MDPCs during adipogenic differentiation. Both siKLB and scrambled siRNA groups were transfected 24 hours prior to induction of differentiation and on day 3 of differentiation. All data are shown as Mean ± SE values from 4 biological replicates, P values shown on graph (B) Representative fluorescent images showing KLB immunostaining (red) in siKLB- or scrambled siRNA- transfected MDPCs on day 2 and day 4 of differentiation. Nuclei were counterstained with DAPI. Scale bar =50µm. I thank Yennifer Cortes Araya for providing data from 3 animals and the images used in this figure.
Figure 4.5 Effects of KLB downregulation on the adipogenic differentiation of MDPCs.

(A) Representative bright-field images (top pane) and ORO staining of intracellular lipids (bottom pane) in siKLB- or scrambled siRNA- transfected MDPCs on day 7 of differentiation. Scale bar =100 µm. (B) Relative expression of PPARγ and FABP4 transcripts in siKLB- or scrambled siRNA-transfected MDPCs during adipogenic differentiation. Data are shown as Mean ± SEM from four independent experiments, P values shown on graphs. I thank Yennifer Cortes Araya for providing data from 3 animals and the images used in this figure.
4.4 Effects of CRISPR/CAS9-aided Functional Deletion of KLB on Adipogenic Differentiation of Porcine MDPCs

4.4.1 Optimization of Transfection in Porcine MDPCs

To test an appropriate CRISPR transfection protocol for use in porcine MDPCs, cells from one animal, were transfected with a plasmid containing the reporter gene, EGFP using either a cationic lipid (Lipofectamine 3000) or by electroporation.

For lipofectamine transfection, pig MDPCs were transfected with either 5 µg or 2 µg of DNA in 10 µl or 4 µl of lipofectamine 3000 reagent respectively and cells were examined 48hrs later. Transfection efficiency was about 10% using 5µg of DNA and 6% using 2µg of DNA (averaged over 3 fields taken from 1 well) as illustrated in representative immune-fluorescence images taken 48 hrs after transfection (Figure 4.6A). Next, the transfection efficiency of same EGFP expressing plasmid was tested using electroporation. To do this, 0.5x10⁵ MDPCs were transfected with 1 µg of DNA using the electroporation system set at 1650v (pulse voltage), 10ms (pulse width) and 3 electrical pulses. As shown in Figure 4.6B, about 60% of the cells showed GFP expression at 48 hours. Thus, electroporation was used in subsequent experiments.
Figure 4.6 Efficiency of DNA transfection into porcine primary MDPCs.

Representative images of pig MDPCs transfected with GFP expressing plasmid using lipofectamine (A) or the Neon™ transfection system (B). Scale bar=100µm.
4.4.2 Generation of CRISPR/Cas9 Vectors

4.4.2.1 Selection of prospective gRNAs

The workflow for generating edits in pig MDPCs is shown in Figure 4.7A. Pig MDPCs were co-transfected with GFP- or RFP- labelled CRISPR/Cas9 vector. Cells expressing both GFP and RFP were enriched by FACs as RFP+/GFP+ and collected as a pool or sorted as single cells into 96 well plates for culturing single cell populations. Both the unenriched and enriched pool of RFP+/GFP+ cells as well the single cell clonal population were analysed for gene editing by gel electrophoresis and sequencing.

The schematic illustration of the KLB targeting strategy is shown in Figure 4.7B. Two potential target sites in exon 2 were identified with a goal to generate a 200bp deletion via co-transfection with a pair of guide RNAs (gRNAs). Also, two genotyping primers were set outside the target site. A 200 bp deletion coupled with the two genotyping primers placed outside the target region allows for ease of genotyping after editing.

To select gRNAs that can be used for genome editing in porcine primary MDPCs in vitro, genomic DNA (gDNA) was extracted from porcine MDPCs. The target site was amplified with PCR primers and the PCR products sequenced. Using the second exon in KLB, the target region was sequenced with four sequencing primers spanning the target region to identify single nucleotide polymorphisms (SNPs) in the target region.
Figure 4.7 **Selection of prospective gRNAs.**

(A) Workflow for gene editing porcine MDPCs. (B) Schematic illustration of porcine KLB deletion strategy with the target region showing potential gRNAs, CRISPR-gRNA target site and genotyping primers.
4.4.2.2 CRISPR/Cas9 Reagent Construction

The publicly available tool, CHOPCHOP (http://chopchop.cbu.uib.no/) (Labun et al., 2019) was used to aid the design and ranking of candidate gRNAs in the target region. Among the candidate gRNAs in exon 2, six gRNAs were selected based on high specificity, minimal predicted off-target Cas9 binding scores. Of the six gRNAs, three gRNA at 5’ and three gRNA at the 3’ end of KLB were inserted into a PX458 vector expressing RFP or GFP respectively. To screen for correct insertion of gRNAs, colony PCR products were purified for Sanger sequencing.

Sanger Sequencing results showed that only 4 (2 at each end) out of the 6 oligos inserted correctly into the vector. These 4 gRNAs (KLB-gRNA-2, KLB-gRNA-3, KLB-gRNA-4, and KLB-gRNA-5) were used for subsequent experiments.

4.4.2.3 Selection of gRNA with High Efficiency

To test the cutting efficiency of the guides, 1 µg of each CRISPR/Cas9-RFP vector (containing KLB-gRNA-2 or KLB-gRNA-3) or CRISPR/Cas9-GFP vector (containing KLB-gRNA-4 or KLB-gRNA-5) was transiently transfected into 50,000 MDPCs using the Neon™ transfection system described in section 4.2.4.1. Negative control was a sample transfected with resuspension buffer. Twenty-four hours after transfection, the cells were observed for fluorescent activity. All transfected wells had cells showing RFP expression (Figure 4.8A) or GFP expression (Figure 4.8B) indicating successful transfection. The negative control showed no GFP expression (Figure 4.8B, -ve control).
To estimate the cutting efficiency, genomic DNA (gDNA) was extracted from the pool of transfected MDPCs and the target regions were PCR amplified and sequenced. The Sanger sequence trace files were analysed by TIDE algorithm (available online at http://shinyapps.datacurators.nl/tide/ (Brinkman et al., 2014b)) (Figure 4.9A). The sequence traces for each gRNA and a paired control is shown in Figure 4.9 B-E while the predicted in/del frequencies are shown in Figure 4.9 f-i. According to the tide assays the four gRNAs tested can
lead to DSB at the target sites accurately but with varying cutting efficiencies. For the gRNAs at the 5’ end of the target region, the reported editing efficiency of KLB-gRNA-3 was 60.7% which was higher than the 32% efficiency of KLB-gRNA-2. Likewise, analysis of the gRNAs at the 3’ end of the target region showed an editing efficiency of 67.2% for KLB-gRNA-4 which was higher compared to the 36.3% efficiency recorded for KLB-gRNA-5. As a result, KLB-gRNA-3 and KLB-gRNA-4 were selected for subsequent experiments.
4.4.3 Deletion of KLB and Effects on Adipogenesis by MDPCs

4.4.3.1 Co-transfection of Pig MDPCs and Isolation of FACS-Enriched Mixed Cell Pool and Single Cell Clonal Populations

Based on the TIDE analysis, two gRNAs (KLB-gRNA-3 and KLB-gRNA-4) were selected to generate an estimated 200 base pair (bp) deletion in exon 2 of KLB (Figure 4.10A). Two concentrations of each gRNA were tested; 1 µg or 0.5 µg of KLB-gRNA-3-RFP plasmid and 1 µg or 0.5 µg of KLB-gRNA-4-GFP plasmid were co-transfected into porcine MDPCs. Twenty-four hours post transfection, RFP+/GFP+ cells were selected as FACS-enriched mixed cell pool or sorted into 96 well plates for single cell clonal growth. For FACS analysis, side scatter area (SSC-A) vs forward scatter area (SSC) dot plots were used to gate healthy cells and exclude non cellular material. These cells were further gated in a forward scatter height (FSC-H) vs forward scatter area (FSC-A) plot to select single cells. Lastly, RFP+/GFP+ cells were selected in a double plot for Texas Red vs eGFP. The FACS plot showed that there was no difference in the light scattering pattern or the size of transfected and non-transfected cells (Figure 4.10 B-C, left and middle panes). As expected, the non-transfected cells did not have any fluorescent activity and were collected.
as wild type cells from the negative gate either as a pool or single cells (Figure 4.10B right pane). Transfected cells on the other hand had both RFP and GFP fluorescence and cell populations with highest RFP/GFP expression in RFP+/GFP+ gate were sorted as either FACS-enriched mixed cell pool or single cells and analysed (Figure 4.10C, right pane).

Figure 4.10 Genetic deletions using two gRNAs.

(A) Diagrammatic sketch of KLB showing gene editing strategy with selected gRNAs and PCR primers for assessing genomic deletion. Two PCR primers (red arrows), spanning the target region were used to amplify a 700bp DNA fragment in WT untransfected cells and a 500bp product in edited cells. FACS analysis of (B) Untransfected MDPCs and (C) MDPCs co-transfected with a PX458 vector expressing SpCas9-2A-GFP-gRNA and a second PX458 vector expressing SpCas9-2A-RFP-gRNA. Events displayed in a dot plot as SSC-A vs FSC-A were gated to exclude noncellular material (B and C, left panel) and further gated to select singlets in FSC-A vs FSC-H plot (B-C, middle panel). Double plot for Texas red and GFP with high GFP and texas red expressing cells gated for selection of edited RFP+GFP+ cells in C (right panel). WT cells were selected from the negative fraction in double plots for Texas red vs GFP in B (Right panel). RFP+GFP+ cells and WT cells were either collected as a mixed cell pool or sorted as single cells into single wells of a 96 well plate.
4.4.3.2 PCR analysis for Loss of Function Genomic Deletions in Mixed Pool

To estimate efficiency of indel mutations in the mixed cell pool, gDNA was isolated from unenriched transfected cell pool, FACS-enriched RFP+GFP+ mixed cell pool and un-transfected wild type mixed cell pool for PCR amplifications. The PCR products were visualised on a 1% agarose gel and results are shown in Figure 4.11A-B. The PCR amplification using the forward and reverse primers shown in Figure 4.10A was expected to yield a 700bps products in the wild type and 500bp product in MDPCs with deleted KLB due to absence of the 200bps target region, assuming each DSB occurs at the expected 3bps upstream of the PAM. The result indicated that 0.5 ug or 1ug of DNA used for transfection each resulted in monoallelic deletions in both the unenriched mixed cell pool (Figure 4.11A) and the FACS-enriched mixed pool populations (Figure 4.11B). As expected, wild type cells as well as non-transfected cell control showed a single wild type band.

To confirm KLB deletion and identify indel mutations, PCR products were sequenced and analysed by inference of CRISPR Edits (ICE) analysis (available online at http://ice.synthego.com (Conant et al., 2022) (Figure 4.11, C-D). The result from the Ice analysis showed that the gRNAs induced a DSB at the target sites accurately. The indel mutations observed was uniform in the FACS-enriched mixed cell pool with 81% showing a 199-200 bps deletions compared to the Un-enriched mixed cell pool which had a more dispersed indel mutations and only 28% had a 199-200 bps deletions. Also, the predicted rates of total indel efficiencies were highest in the FACS-enriched mixed cell pool reaching 82% compared to 48% in the un-enriched mixed cell pool. These results indicates that despite the high transfection efficiency observed, FACS is an important step in purifying positively edited porcine MDPCs. Thus, only the FACS-enriched mixed cell pool population was used for subsequent downstream experiments.
Figure 4.11 Analysis of genomic deletions using two gRNAs in mixed cell pool.

(A) PCR analysis of CRISPR-Cas9-mediated genomic deletions in KLB gene in unenriched mixed cell pool 24 hours after transfection prior to FACS. (B) PCR analysis of CRISPR-Cas9-mediated genomic deletions in KLB gene in FACS-enriched mixed cell pool 24 hours after transfection. The size of the WT PCR products is shown in the upper lane and are 700 bps. The anticipated band for the deleted amplicons is approximately 500 bps corresponding to the lower bands. (C-D) Analysis of the editing quality of gRNAs by ICE software in silico. (C) Representative discordance graph of Sanger sequence traces from PCR amplified wild type cells (in orange) and targeted cells (in green). The alignment window (red bar) corresponds to the alignment region between the control and target traces, and the inference window (gray bar) corresponds to the region around the predicted sites of genome editing. (D) Quantification of the indel efficiency in FACS-enriched and unenriched mixed cell pool where 0 represents no editing (wild type); indel mutations are presented as “−” (deletion) or “+” (insertion). The predicted total percentages of indel mutations are indicated on the left.
4.4.3.3 **PCR Analysis for Loss of Function Genomic Deletions in Single Cell Clonal Populations**

Out of the 192 edited clones sorted, only 11 clones successfully grew after 6 weeks of culturing and were analysed. As with the mixed cell pool, gDNA was extracted from each clone and PCR amplified. The PCR products were visualised in a 1% agarose gel (Figure 4.12 A). The expected PCR product was 700bps in the wild type and 500bp product in the deleted *KLB* due to absence of the 200bps target region assuming each DSB occurs at the expected 3bps upstream of the PAM. Of the 11 clones analysed, 6 had apparent biallelic deletions, 3 contained monoallelic deletions while the remaining 2 had no apparent deletions but may contain indels.

The purified PCR products from selected clones (Clones 1, 4, 6 and 9) were sequenced to confirm deletions. As shown in Figure 4.12B, MAFTT alignment of the Sanger sequence traces confirmed the deletions. Due to the time and financial constraint, these clones were not further analysed.
Figure 4.12 Analysis of genomic deletions using two gRNAs in single cell clonal populations.

(A) PCR analysis of CRISPR-Cas9-mediated genomic deletions in KLB in single-cell clonal populations. The size of the WT bands is shown in the upper lane and are 700bps. The anticipated band for the deleted amplicons is approximately 500bps corresponding to the lower bands. (B) Sequence alignment of Sanger sequencing traces from PCR products for KLB-Clone 1, KLB-Clone 4, KLB-Clone 6 and KLB-Clone 9 showing the gRNAs used and the deleted region. MAFFT alignment obtained from Lasergene MegAlign Pro 14 (DNASTAR).
4.4.3.4 Adipogenic Responses in KLB-deleted MDPCs

To assess the effect of KLB deletion on the ability of MDPCs to differentiate into adipocytes, wild type MDPCs (WT-MDPCs) and FACS-enriched KLB knockout MDPCs (KLB-KO) were seeded on collagen and stimulated to differentiate in the presence of IBMX as described in section 2.5.6. The results showed that both WT and KLB-KO were able to accumulate lipid droplets and form adipocytes (Figure 4.13 A). However, differentiation was more efficient for WT-MDPCs than for KLB-KO MDPCs as evidenced by an adipocyte count of 9±0.6 vs 1.5±0.3 per field in WT-MDPCs compared to KLB-KO cells. Also, the expression of the fatty acid carrier protein FABP4 was higher in WT-MDPCs compared to KLB-KO cells on days 7 and 14 of differentiation (Figure 4.13B). The results showed that the level of KLB transcript increased as differentiation progressed in the wild type cells but not in KLB-KO cells indicating a loss of expression in KLB-KO cells (Figure 4.13C).
Figure 4.13 Adipogenic differentiation of wildtype and edited MDPCs.

(A) Representative bright-field images of wildtype and edited MDPCs cultured under adipogenic conditions for 14 days. Arrow heads indicate adipocytes. Scale bar =100 µm and 50 µm for insert (B-C) Level of expression FABP4 and KLB transcripts on days 0, 7 and 14 of adipogenic differentiation. Values are shown as fold change expression relative to Day 0 values. Data shown as Mean ± SE values from triplicate wells of a single experiment.
4.5 Discussion

Previous work at Roslin demonstrated an involvement of FGF21/KLB signalling in impaired muscle development in IUGR pigs (Cortes - Araya et al., 2022), however whether this pathway may be involved in the increased propensity of muscle from IUGR pigs to accumulate fat was not investigated. Using different approaches including RNAi mediated KLB knockdown and CRISPR/Cas9-mediated deletion of KLB, the results presented in this chapter demonstrate the involvement of FGF21-KLB in regulating adipogenic differentiation of porcine MDPCs. In addition, this study demonstrates the value of CRISPR/Cas9- mediated genome editing for studying gene function in porcine MDPCs.

4.5.1 FGF21 Stimulation Enhances Adipogenesis by Porcine MDPCs

FGF21 has been characterised as a potent metabolic regulator involved in lipid and glucose metabolism with a potential to treat disease conditions associated with insulin resistance (Kharitonenkov et al., 2005, Kharitonenkov et al., 2007). However, the observed effect of FGF21 on lipid metabolism in adipocytes have been inconsistent, with in vitro studies having reported both lipogenesis (Arner et al., 2008) and lipolysis (Inagaki et al., 2007). For example, studies in FGF21 KO mice showed that FGF21 increased lipolysis in white adipose tissue of animals fed normally but inhibited lipolysis in fasted mice (Hotta et al., 2009).

Consistent with the results of the present study with porcine MDPCs (Figure 4.3), previous studies showed that FGF21 increased adipogenic differentiation of human MSCs and mouse 3T3-L1 adipocytes in a dose-dependent manner (Arner et al., 2008, Moyers et al., 2007). Similarly, treatment with FGF21 increased differentiation and lipid accumulation by human abdominal subcutaneous pre-adipocyte (Berti et al., 2015). FGF21 has also been shown to attenuate hormone-stimulated lipolysis in both human and murine adipocytes (Arner et al., 2008). Furthermore, other studies have shown that
treatment with FGF21 inhibited adipocyte lipolysis in both mouse primary adipocytes and in differentiated 3T3-L1 adipocytes \textit{in vitro} (Li et al., 2009) and \textit{in vivo} (Chen et al., 2011, Li et al., 2009). Taken together, these data suggests that, as in other species, FGF21 signalling can regulate adipogenesis in porcine MDPCs.

In contrast to my findings, over expression of FGF21 in intramuscular pre-adipocytes significantly reduced lipid accumulation and down-regulated the expression of key adipogenesis regulatory genes, peroxisome proliferator-activated receptor gamma (PPARG), CCAAT/enhancer-binding protein (CEBP) family and adipocyte fatty acid-binding protein 2 (aP2) in pig (Wang et al., 2015) and goat (Xu et al., 2021). Differences in the stability of FGF21 may have accounted for the discrepancies in results between those studies and mine. Thus, whereas they overexpressed FGF21 by using adenovirus mediated stable transfection I used supplementation of culture media with FGF21. Also, FGF21 shows a propensity to form aggregates \textit{in vitro} in a manner that depends on concentration, temperature and time (Hecht et al., 2012), and formation of such aggregates may limit the availability of FGF21 and thus account for the differences observed in those \textit{in vitro} studies.

\textbf{4.5.2 Loss of Expression of KLB inhibits Adipogenesis by Porcine MDPCs}

In agreement with the results that KLB expression increases during adipogenic differentiation of porcine MDPCs, I found that KLB Knockdown (using siRNAs) or knockout (using CRISPR/Cas9) suppressed MDPC differentiation. Consistent with these results, Phelps et al. (2016a) reported that \textit{KLB} expression increased in mice fibro/adipogenic progenitors (FAPs) undergoing adipogenic differentiation, while overexpression of \textit{KLB} enhanced adipogenesis in NIH3T3 fibroblasts. However, \textit{KLB} had no effect on differentiation of isolated mouse satellite cells or C2C12 myogenic cells. In addition, a study on dystrophin-null mice model reported that \textit{KLB} expression was upregulated in diaphragm muscle concomitant with the pathology of
enhanced fibrosis and adipogenicity in muscle (Stuelsatz et al., 2012a). Also, recent work from our group showed that siRNA mediated knockdown of KLB promoted myogenesis whereas treatment with FGF21 decreased myogenesis suggesting a role of KLB in impaired muscle development (Cortes-Araya et al., 2022). Taken together, these findings suggests that FGF21 signalling through KLB promotes skeletal muscle adipogenesis while inhibiting myogenesis and may also be involved in pathological fibro/adipogenesis in skeletal muscle.

4.5.3 CRISPR/Cas9 can be used for Successful Targeted Genetic Deletion in Porcine MDPCs

In predicting gRNAs for a CRISPR/Cas9 study, one concern is the presence of potential SNPs in the target region as the reference sequence may be different from the sequence of the cell line under study (Kim et al., 2021). This was a particular concern in my study due to the significant genetic diversity in the pig population. Using several sequencing primers spanning the length of target exon 2 of KLB, I revealed that there were no SNPS in the target region in porcine MDPCs. Although there were regions with poor quality sequence traces, gRNAs were selected in regions without mismatches. The consequence of the presence of SNPS in the target region is that the binding of the gRNA may be impaired or even prevented (Vochozkova et al., 2019). Thus, it is imperative to sequence verify the target region in the cells before designing the gRNAs.

The ability of predicted gRNAs to effectively direct Cas9-mediated DNA modifications varies. Therefore, to avoid poor genome editing outcomes, gRNA activity should be validated by quantifying the modification frequencies prior to its use in the actual genome editing experiment. Assays commonly used to assess level of activity of a gRNA include T7 endonuclease 1 (T7E1) (Mashal et al., 1995), indel detection by amplicons analysis (IDAA) (Yang et al., 2015), TIDE-coupled Sanger sequencing (Brinkman et al., 2014a), ICE-coupled sanger sequencing (Conant et al., 2022) and Next generation
sequencing (NGS) (Bell et al., 2014). In this study, Sanger sequencing coupled with TIDE and ICE analysis were used to validate the cleavage efficiency of individual gRNAs. Targeted NGS provides accurate estimates of indel sizes, frequencies and sequence identity in edited cell pools and clones making it the optimal tool for validating gRNA activity (Sentmanat et al., 2018). However, it is not widely available, and the high cost and labour required to set it up makes it prohibitive for small sample sizes. While T7E1 assay is a simple, cost-effective and easy to interpret gRNA validation technique, the estimated nuclease activity most often differs from the activity observed in edited cells compared to NGS (Sentmanat et al., 2018). Both TIDE, ICE and IDAA are cost-efficient alternatives that have been shown to predict editing efficiencies for mixed pools of cells at a rate comparable to targeted NGS (Hsiau et al., 2019, Sentmanat et al., 2018). By combining ICE with TIDE analysis to assess gRNA activity in this study, I was able to select gRNAs that gave consistently high cleavage activity on both platforms. Similarly, Sanger sequencing coupled with either TIDE or ICE analysis have been used to detect mutations post editing in several species (Kim et al., 2021) including the pig (Zhang et al., 2019).

I generated KLB-KO MDPCs through co-delivery of dual CRISPR/Cas9 complexed gRNAs to induce a 200bp deletion that would result in frameshift mutation in the coding sequence of the gene. One major advantage of this strategy is the ease of genotyping. Using spanning PCR and Sanger sequencing coupled with ICE analysis, I showed both monoallelic and biallelic deletions in mixed cell pools as well as single cell clones. This strategy has been used to knock out various genes in several species including Pig ST6GAL1 (Du, 2019). It must be noted that the use of CRISPR/Cas9 may result in the creation of off-target mutations in unintended genomic regions (Cho et al., 2014). To prevent this, I used an optimised online gRNA selection programme, CRISPOR.tefor.net, to select rationally designed gRNAs with minimal off-target activity (Concordet and Haeussler, 2018). The potential off-target sites listed for the selected gRNAs used in this study were not assessed, however, all the predicted off-target sites contained Cas9-blocking mutations.
in either the PAM or gRNA seed sequence that are likely to prevent off-target Cas9 cleavage (Jinek et al., 2012a, Hsu et al., 2013). Future studies should perform sequencing of predicted off-target sites where possible to confirm the absence of non-specific CRISPR-induced mutations that may alter cellular activity.

In this study, validation of loss-of-function deletion of KLB in pig MDPCs was done by PCR coupled with sanger sequencing. In addition, RT-qPCR was performed to measure KLB mRNA levels to show loss/ reduced expression. The NHEJ repair technique as used in the study leads to nonsense-mediated mRNA decay resulting on mRNA degradation. Nonsense-mediated mRNA decay is caused by the introduction of a premature termination codon, which results in the production of a truncated protein product or mRNA degradation and loss of expression (Popp and Maquat, 2016, Lindeboom et al., 2019). Lastly, the loss-of-function phenotype was analysed in vitro by comparing the adipogenic differentiation potential of KLK-KO line and the wildtype cells. Future studies should include assessment and quantification of KLB protein expression levels using either Western blotting, immunohistochemistry, or enzyme-linked immunosorbent assay (ELISA) to help validate the gene editing. In CRISPR Cas 9 experiments, the target gene is often disrupted, and the protein expression levels of the target gene should be significantly reduced or absent if the editing is successful. Therefore, protein expression levels provide direct evidence of Cas 9 activity and the efficiency of gene editing, help identify off-target effects, and reveal the functional consequences.

Like their human counterparts, porcine myogenic cells have limited proliferative capacity due to cellular senescence in culture, and loose stemness following prolonged culture (Ding et al., 2017). Particularly, single cell clones derived from primary muscle stem cells are difficult to propagate in vitro as they have to go through a large number of population doublings in order to withstand selection and expansion (Benedetti et al., 2018b). Therefore, sometimes it is only possible or more convenient to study gene function in cell pools rather than in clonally isolated cell lines. In my study, growing MDPCs as single cells proved difficult due to very low survival rate
and long propagation time in vitro. Therefore, it was not possible to use these clones for downstream experiments following confirmation of deletions. In addition to replicative senescence triggered by excessive shortening of the telomeres, suboptimal cell culture conditions can result in premature growth arrest in muscle stem cells (Zhu et al., 2007a). Human myogenic cells have been immortalized by introduction of human telomerase reverse transcriptase (hTERT), hTERT and Bmi1 or hTERT and Cyclin dependent Kinase 4 (CDK4) transgenes as a means of extending proliferation capacity and maintain stemness and regenerative capacity upon transplantation. Following successful immortalisation, human myogenic cells could be clonally isolated and expanded in large scale for both therapeutic and research purposes (Benedetti et al., 2018b, Hashimoto et al., 2006, Au - Robin et al., 2015, Cudré- Mauroux et al., 2003, Zhu et al., 2007a). Therefore, it may be useful to immortalise porcine MDPCs to allow for extended proliferation and clonal analysis as a tool for genetic engineering and gene function studies.

4.6 Conclusion

This chapter provides proof-of-concept of the value of the in vitro skeletal muscle model described in chapter 3 for studying muscle function in the pig. Using this model, I showed that FGF21-KLB signalling promotes skeletal muscle adipogenesis. While the precise mechanisms involved is yet to be elucidated, it is likely that this involves FGF21/KLB activation of adipogenic pathways in muscle resident FAPs or mesenchymal stem cells (MSCs) (other than satellite cells) (Judson et al., 2013). However, there is a need to further investigate involvement of the FGF21-KLB in the dynamics of fibro/adipogenesis in skeletal muscle to elucidate the molecular and cellular changes associated with enhanced fibrosis and fat infiltration in muscle pathologies and aging.

Despite the intrinsic limitations of working with primary cells, my results demonstrate that CRISPR/Cas9 genome editing can be used to study specific genes in porcine MPDCs. Moreover, the technical and procedural route for
utilising CRISPR/Cas9 gene editing tool in porcine MDPCs was optimised which could provide reference for future studies. However, the CRISPR/Cas9 results from this study must be interpreted with caution. First, cells from a single animal were edited, therefore the wider applicability of this protocol will need to be established by using cells from additional individuals. Secondly, due to time and project funding constraints, the phenotypic analysis (adipogenic differentiation potential) was carried out in pooled cells rather than single cell clones, with the risk that wild type cells in the pool may have interfered with testing of the effects of KLB knockout. Therefore, there is need to verify these results in single cell clones.
Chapter 5 General Discussion and Future work

The ever-increasing human population, coupled with rising affluence in developing countries, will necessitate an increase in global meat production to meet protein demands whilst minimising the environmental impact of farming. According to FAO estimates, meat production needs to increase by 76% to meet demand. (Alexandratos and Bruinsma, 2012). Although plant-based alternative proteins are becoming more popular, their ability to address the issue is limited by long-term dietary deficiencies and consumer preferences for meat (Stephens et al., 2018).

Skeletal muscle from agricultural livestock provides a high-quality protein source, and an increase in meat production would necessitate a better understanding of muscle growth and development in agricultural livestock. Being a multiparous species able to produce up to 40 offspring per female a year, the pig provides a potentially very efficient source of meat. Growth efficiency is closely related to the proportion of lean to fat tissue in the body. Previous studies have looked at the effects of genetics, feed nutrition, housing conditions, and breeding management on pig production variables such as body weight, feed utilisation efficiency, meat quality, and reproductive success (Mellencamp et al., 2008). However, more research focusing on understanding the biological drivers of muscle growth is essential for devising efficient strategies to maximise meat production in pigs.

Harnessing muscle stem cells has great potential for studying muscle illnesses and for furthering our knowledge of muscle growth and meat production. For example, muscle stem cells are widely used in model systems to better understand muscular myopathies, and in tissue engineering and drug discovery (Cerletti et al., 2008, Wallace et al., 2008, Vandenburgh et al., 2008, Mierzejewski et al., 2020a). In addition, muscle progenitor cell populations are utilised in stem cell transplantation as a cell-based therapy towards restoring muscle function in diseases such as Duchenne muscle dystrophy (DMD)
Specific populations of muscle-derived stem cells have proven their potential in the treatment of myopathies (Cerletti et al., 2008). The same is true in the field of animal and meat science where stem cell research focuses on characterising and cultivating adipogenic and myogenic progenitors for the purposes of increasing meat production and, more recently, generating in vitro grown meat (Dodson et al., 2015, Dohmen et al., 2022).

This thesis focuses on establishing and characterising a robust system to isolate and culture porcine myogenic and adipogenic progenitor cells. Among other applications, the ability to robustly isolate and culture porcine progenitor cell populations would provide an in vitro platform for studying the biology underlying pig skeletal muscle development and meat production and perhaps even enable large-scale cell production for cultured porcine meat applications.

5.1 Significance of the Findings in this Study

5.1.1 CD146 Marks Myogenic Progenitor Populations Within Pig Skeletal Muscle.

Various myogenic progenitors present in mouse skeletal muscle have been identified, some of which can be isolated using cell surface markers and fluorescence activated cell sorting (FACS) (Péault et al., 2007, Mierzejewski et al., 2020a). These cell surface markers are well characterised in mouse and humans but not so in pigs. In chapter 2, I describe the isolation and characterisation of two distinct progenitor cell populations from porcine muscle by FACS on the basis of positive or negative expression of CD146. In this experiment I showed that although both CD146+ cells and CD146- cells display adipogenic differentiation potential in vitro, CD146+ cells were distinctly myogenic in vitro indicating that CD146+ cells contain myogenic progenitors while the CD146- cells contain adipogenic progenitor cells. This result indicates that CD146 can be used as marker for the isolation of myogenic progenitor cells from pig muscle as reported for human foetal muscle.
Surface markers for muscle stem cells in pig are not well characterised. Besides CD56, two other surface markers, CD29 (Ding et al., 2017, Choi et al., 2020a) and integrin α6 (Wilschut et al., 2011) have reportedly been used to isolate myogenic cells from porcine muscle. My finding provides additional information on the biochemical properties of pig myogenic cells and adds CD146 to the list of potential surface markers of porcine myogenic cells thereby expanding the field of knowledge.

Notably, work in chapter 2 reveals the possibility of concurrently isolating myogenic and adipogenic progenitors using either CD56 or CD146. This finding provides information regarding the diversity of muscle stem populations with distinct lineage commitments implying a hierarchy in muscle tissue. In addition, the CD146+ cells showed multipotency in vitro capable of differentiating into myogenic and adipogenic lineages under the appropriate conditions. An approach, which would broaden the usefulness of these cells, would be the establishment of a muscle and fat co-culture system. Hypothetically, these cells could be used to develop such a co-culture system either in a single plane to study the effect of physical contact on cell function and lineage specificity or alternatively on separate surfaces to study the effects of soluble cell-specific secretions on adjacent cell types (Pandurangan and Kim, 2015, Dodson et al., 1997). Such co-culture system could facilitate further understanding of crosstalk between different tissue progenitor populations during myogenesis and adipogenesis and provide useful information on improving pig meat production. Additionally, it could be used to study pig muscle growth and repair mechanisms as well as muscle-related diseases including DMD and obesity (Dodson et al., 1997). Co-culturing myoblasts and macrophages, for instance, has allowed researchers to study the interplay between these two cell types; this has implications for muscle regeneration and repair (Stewart et al., 1997, Cantini et al., 1994, Cantini and Carraro, 1996). Furthermore, myogenic cells and fibroblasts were co-cultured in a contact system, shedding light on the mechanisms that regulate skeletal muscle development. In this co-culture technique, fibroblasts specifically
surrounded the differentiated myotubes with extracellular matrix, allowing the cells to create a functional, contracting 3D muscle structure (Strohman et al., 1990).

In addition, a muscle and fat cell co-culture system will be particularly beneficial in the growing field of cultured meat. Current research to improve cultured meat production is focused on optimising the culture conditions that will allow for in vitro expansion of muscle cells. However, the production efficiency and ability of in vitro meat to replicate conventional meat attributes, such as the presence of intramuscular fat (marbling), which is crucial for giving meat flavour, texture, and juiciness, will determine how well it is accepted (Verbeke et al., 2015, Tomiyama et al., 2020, Jairath et al., 2021). In this regard, differentiated C2C12 and 3 T3-L1 cells were co-cultured to investigate the effect on fat and muscle formation, and it was demonstrated that the expression of myogenic and adipogenic marker genes was significantly altered in co-cultured C2C12 and 3T3-L1 cells when compared to their respective monoculture controls (Muthuraman, 2014). Also, in order to investigate the potential interaction between muscle and fat cells, 3T3-L1 preadipocytes and satellite cells were co-cultured. The results demonstrate that satellite cells may secrete substances like insulin-like growth factor-1 and insulin-like growth factor-binding proteins that affect the viability and differentiation of preadipocytes. (Dodson et al, 1977). Furthermore, Muthuraman and colleagues focused on establishing successful co-cultures of a variety of cells types including C2C12, 3T3-L1 cells, myoblast, fibroblast cells and muscle tissue and embryo in order to study animal fat regulation and muscle deposition (Pandurangan and Kim, 2015). Understanding the cross talk between muscle and fat systems during myogenesis and adipogenesis could be utilised to improve the quality of in vitro grown meat.

In addition to the co-culture experiments outlined above, future work could also examine the in vivo engraftment potential of porcine muscle-derived CD146+ cells. Previous study reported that MCAM+ cells isolated from human foetal myoblast were capable of engraftment in vivo following transplantation into immune compromised mice and regenerate dystrophin expressing fibres.
(Lapan et al., 2012a). Similarly, CD56+/CD29+ satellite cells isolated from pig muscle were reportedly able to engraft and contributed to muscle regeneration following transplantation into immune compromised mice with muscle injury induced by cardiotoxin injection. In addition, the transplanted cells were able to home to the satellite cell niche in recipient mice (Ding et al., 2017). Future research could look into the ability of porcine muscle-derived CD146+ cells to engraft in vivo as well as their ability to home in the correct niche. The results of these investigations will increase the potential of these cells for cell-based-therapy models to develop treatments for muscle diseases including Duchenne muscular dystrophy.

5.1.2 Explant Culture Enables Efficient and High Yield Derivation of Myogenic Cells from Porcine Skeletal Muscle

Chapter 3 describes the derivation and characterisation of a novel explant culture from porcine skeletal muscle. There, I report a protocol that utilises a culture medium with high serum and bFGF in combination with matrigel coating to isolate myogenic progenitor cells by encouraging their migration from their natural niche. Further characterisation revealed that the muscle derived progenitor cells can be expanded robustly in vitro and sustain long-term proliferation in vitro. In addition, the MDPCs obtained from this method expressed key muscle stem cell markers and were able to efficiently form myotubes in vitro. Furthermore, after prolonged culture, the MDPCs lost expression of muscle stem cell markers, decreased myogenic potential, and increased Mesenchymal Stem Cell (MSC) marker expression as well as adipogenic potential.

Extrinsic cues, such as soluble secreted molecules and local extracellular matrix (ECM) proteins, are used by the stem cell niche to control the differentiation and fate of muscle progenitor cells in vivo. Previous studies have incorporated niche associated components like growth hormones and ECM proteins to help maintain the myogenic and self-renewal capabilities of embryonic stem cells (Villa-Diaz et al., 2010, Melkoumian et al., 2010, Rodin
et al., 2010) and adult muscle stem cells (Gilbert et al., 2010). Using matrigel in combination with high serum media supplemented with basic fibroblast growth factor (bFGF), I was able to obtain porcine MDPCs that expressed PAX7 and MYOD and displayed an efficient myogenic potential. Matrigel is composed mainly of laminin which supports the migration and proliferation of mice and human myoblast while maintaining their myogenic phenotype during prolonged culture in vitro (Penton et al., 2016). Matrigel used in combination with bFGF has been shown to support the outgrowth of muscle stem cells with high myogenic efficiency in other species (Wang et al., 2014, Shahini et al., 2018, Marg et al., 2020). However, explant culture using muscle fragments on gelatin or uncoated flasks gave rise to progenitor cells capable of differentiating into chondroblasts, osteoblasts and adipocytes but not myocytes indicating that the cells obtained were mesenchymal stem cells and not myogenic progenitor cells (Ceusters et al., 2017, Wilschut et al., 2008). The explant culture protocol described in Chapter 3 aided the isolation of highly myogenic progenitor cells that could be expanded rapidly from pig and can be applied to other meat producing livestock like cow which can be utilised for the study of muscle development and increasing meat production efficiency as well as for scalable in vitro cultured meat production.

I was able to obtain millions of minimally differentiated myogenic progenitors that retained in vitro myogenic differentiation potential up to the 8th passage by following the explant method. Because of their high rate of proliferation in vitro, these cells can be used in analyses that normally need a very large sample size, such as western blotting. Growth efficiency is positively related to lean body, i.e. the proportion of skeletal muscle relative to body fat, which can be harnessed to increase meat production directly through selective breeding (Xue et al., 2022). Thus, understanding of key genes and gene variants involved in pig skeletal muscle development would aid understanding of the molecular regulation of muscle growth which is key to efficiently increase meat production. Genome-wide association studies (GWAS) and RNA sequencing analyses have identified several variants associated with muscle growth/development, meat production
and meat quality in pigs (Mohammadabadi et al., 2021, Tang et al., 2019, Ruan et al., 2021). For instance, Fan et al. (2011) conducted a GWAS on 820 commercial female pigs and reported several novel candidate genes that clustered into categories related to muscle growth and development, the insulin pathway or bone and cartilage development. These genes included Bone Morphogenetic Protein 2 (BMP2; for loin muscle area and body size), coiled-coil-helix-coiled-coil-helix domain containing 3 (CHCHD3; for back fat), and some Homeobox A Cluster (HOXA) family genes (for bone and cartilage development). Furthermore, Puig-Oliveras et al. (2022) used expression GWAS (eGWAS) in an Iberian x Landrace intercross population and identifies Insulin Like Growth Factor 2 (IGF2), Monoglyceride Lipase (MGLL), Melanocortin 2 Receptor (MC2R), Rho GTPase activating protein 6 (ARHGAP6), and Nuclear Receptor Subfamily 3 Group C Member 1 (NR3C1) as candidate genes and genetic variants affecting intramuscular fatty acid content and composition in pork. Porcine MDPCs will be an effective in vitro tool for functional studies to verify the value of these candidate genes as markers for pig selection programmes by elucidating their involvement in muscle growth and meat production.

The porcine MPDCs I described in my studies could also be utilised for cellular agriculture purposes. Cells used to produce cultured meat must be fast-dividing to enable the level of scaling-up required for this (Kruglinski and Wright, 2008). To this end, the porcine MDPCs may be suitable for a large-scale cultured meat production procedure since they undergo numerous population doublings in vitro.

The low survival rate of muscle-derived stem cells after transplantation, driven in part by cell death, has been a major constraint in regenerative studies (Guerette et al., 1997, Fan et al., 1996). Consequently, when developing cell-based approaches to treat muscle illnesses, the ability to obtain large amounts of cells (as demonstrated by the porcine MDPCs) for injection is necessary, in order to achieve greater regeneration. Thus, the porcine MDPCs could potentially be utilised to develop cell therapy models. However, their usefulness for regenerative medicine studies will depend on their ability to
demonstrate in vivo engraftment potential. In a previous study, Wang and colleagues used explant cultures to obtain a large number of murine muscle progenitor cells (mMPCs) and demonstrated that the expanded mMPCs when injected into a nude mice's tibialis anterior muscle engrafted and formed myofibres (Wang et al., 2014). Also, satellite cells from cultured human muscle fibre fragments were able to contribute to muscle regeneration after engraftment into mice (Marg et al., 2020). Future research could look into the porcine MDPCs’ ability to participate in skeletal muscle growth, maintenance, and regeneration in response to injury and disease in vivo. In addition, future studies could explore the in vivo systemic migratory potential of the MDPCs and also explore methods to improve their migratory potential to facilitate systemic delivery. Currently, the inability to deliver myoblasts systemically through the circulation is a major hurdle for the use of satellite cell transplantation in the treatment of muscle disorders (Cossu and Sampaolesi, 2007, Patridge, 2000). Overall, if the porcine MDPCs described in Chapter 3 are shown to have sufficiently robust in vivo regenerative potential, they could be a potential candidate for cell-based therapy.

5.1.3 FGF21-KLB Signalling Promotes Skeletal Muscle Adipogenesis

Using both gain and loss of function approaches in porcine MDPCs, my results in chapter 4 demonstrate a role of FGF21-KLB signalling in promoting skeletal muscle adipogenesis. FGF21 has significant functions in regulating lipid and glucose metabolism in the presence of its co-receptor KLB, with KLB restricting the tissue-specific activity of FGF21 to tissues where KLB and FGFRs are specifically co-expressed (Kurosu et al., 2007, Ogawa et al., 2007, Adams et al., 2012). Several studies have revealed that the biological effects of FGF21 on body weight and circulating glucose and insulin levels are mediated primarily through FGFR–bKlotho complex in adipose tissue (Adams et al., 2012; Foltz et al., 2012). While circulating levels of FGF21 in normal muscle are almost undetectable, multiple studies have found that FGF21 levels in
skeletal muscle are elevated in situations of systemic stress, such as hunger, and in some pathological diseases, such as mitochondrial myopathies. (Keipert et al., 2014, Ost et al., 2016, Oost et al., 2019, Vandanmagsar et al., 2016). In addition, it has been reported that serum FGF21 levels are positively correlated with age-related sarcopenia (Vandanmagsar et al., 2016, Hanks et al., 2015) and altered muscle phenotype in growth restricted pigs (Cortes-Araya et al., 2022). Furthermore, the fact that in vivo overexpression of FGF21 in skeletal muscle fibres is sufficient to stimulate autophagosome production and muscle atrophy, resulting in a decrease in the cross-sectional area of transfected TA fibres, supports a role for FGF21 in skeletal muscle remodelling (Oost et al., 2019). These observations suggests that FGF21 may have detrimental consequences for normal muscle growth and development.

As described in chapter 4, treatment with FGF21 increased adipogenesis whereas downregulation of KLB supressed adipogenesis in porcine MDPCs. Studies in genetically modified animals revealed that the amount of total fat mass, adipocyte size, and PPARy activity in white adipose tissue were all significantly lower in FGF21-KO mice. Additionally, FGF21-KO adipocytes displayed decreased lipid formation and decreased expression of lipogenic genes indicating a role in promoting lipogenesis in target tissues (Dutchak et al., 2012b).

There is substantial evidence to support a role of FGF21 in promoting adipogenesis and inhibiting muscle and bone growth. In bone marrow-derived mesenchymal cells from dystrophin/utrophin double-knockout mice, FGF21 was found to increase adipogenesis while concurrently decreasing osteogenesis (Li et al., 2020). In vitro, FGF21 suppressed the growth and differentiation of chondroblasts and osteoblasts while promoting the growth of bone marrow precursors to adipocytes rather than myocytes (Kubicky et al., 2012, Wei et al., 2012). Also, transgenic mice over expressing FGF21 exhibit reduced bone mass, bone mineral density as well as increased number and area of bone marrow adipocytes. Similarly, KLB was increased in muscle of intrauterine growth restricted pig and in skeletal muscle of MDX mice (a mice
model of DMD) where increased levels of KLB have been associated with impaired muscle development characterised by enhanced fibro/adipogenesis and reduced content in lean tissue (Cortes-Araya et al., 2022, Stuelsatz et al., 2012b). Taken together these observations support a role of FGF21-KLB signalling in promoting skeletal muscle fibro/adipogenesis and bone loss.

Fibroadipogenic progenitors (FAPs) and mesenchymal stem cells (MSCs) in skeletal muscle are the known sources of intramuscular fibro/adipogenesis (Judson et al., 2013). Therefore, while the precise mechanisms involved is yet to be elucidated, it is likely that FGF21 and KLB selectively acts on muscle resident FAPs to influence fat formation within muscle. In support of this, increased expression of KLB was observed in mice FAPs but not mouse satellite cells or C2C12 myoblasts undergoing adipogenic differentiation. Also over expression of KLB resulted in increased adipogenesis of NIH3T3 fibroblasts but had no effect on C2C12 myoblasts indicating that KLB acts selectively on FAPs and not satellite cells (Phelps et al., 2016b). In addition, FAP turnover through apoptosis and subsequent clearance from the site of injury promotes muscle regeneration and prevents fibrosis in chronic muscle injury. Failure of FAPs to enter a senescent state after chronic muscle injury reportedly results in accumulation of FAPs and muscle degeneration indicating that FAPs play a role in pro-fibrotic phenotype in chronic myopathies (Saito et al., 2020).

Previous work in the Donadeu lab using porcine and human MDPCs demonstrated that FGF21-KLB signalling suppresses myogenesis and plays a critical role in impaired muscle development (Cortes-Araya et al., 2022). My results in this study supports a pro-adipogenic role of FGF21-KLB signalling in promoting skeletal muscle fibro/adipogenesis. Importantly, my results are consistent with the observed tendency of IUGR piglets to accumulate fat including in muscle, which may be mediated by FGF21-mediated programming of stem/progenitor cell fat in utero (Sarr et al., 2012, Zhang et al., 2022, Li et al., 2015b). There is also evidence that IUGR foetuses contain higher intramyofibrilar fat which may or may not be mediated by high FGF21/KLB
activity in those animals (Karunaratne et al., 2005). As a result, this study provides increased understanding of the role of FGF21-KLB in impaired muscle development. Further research could focus on investigating the effects of FGF21 and KLB on fibrosis of porcine MDPCs. Such studies will expand the usefulness of porcine MDPCs to better understand the role of FGF21-KLB in fibro/adipogenic infiltration associated with the functional deterioration of skeletal muscle in ageing and muscular dystrophy and the characteristically reduced lean tissue content and enhanced fibro/adipogenesis in IUGR muscle.

5.2 Future Studies

5.2.1 Proteomic Analysis and Single-cell RNA Sequencing

I showed in chapter 3 that porcine MDPCs underwent a reduction in myogenic potential simultaneous with an increase in adipogenic potential during in vitro culture. The reason for this change was not unravelled in that study. Skeletal muscle cells produce different types of myokines during proliferation and differentiation in vitro (Ojima et al., 2014). These myokines are thought to play a role in cross talk between myocytes and adipocytes as revealed by studies using co-culture models (Li et al., 2014, Dietze et al., 2002, Li et al., 2017a). According to a proteomic analysis of murine skeletal muscle cells, proliferating myocytes secrete myokines that inhibit neurogenesis and adipogenesis, whereas differentiating myocytes secrete myokines that promote myotube formation, neurogenesis, and vascularization. (Ojima et al., 2014). Future studies could analyse the secretome of porcine MDPCs during in vitro culturing. Proteins could be concentrated from cells or conditioned media at different passages and the proteome analysed using isobaric tagging for relative and absolute quantitation (iTRAQ) technique followed by mass spectrometry (Kim et al., 2012) to quantify differentially expressed proteins. The dynamics of proteins secreted should also be evaluated using Gene ontology (GO) and protein-protein interaction (PPI) analysis to classify the differentially expressed proteins and determine the protein interactome. Furthermore, comparative analyses of selected proteins should be carried out.
to reveal any potential correlation between their mRNA and protein levels in order to provide information on post-transcriptional regulation. These studies will provide valuable insight into changes in protein expression levels, protein stability, their subcellular localization, post-translational modification, and protein interactions within porcine MDPCs during in vitro propagation, which may in turn provide information on the changes in lineage plasticity observed in this study. Furthermore, such research can be used to identify new myokines that mediate the communication between muscle and adipose cells.

In addition to the proteomic analysis described above, future studies could perform a comprehensive single cell analysis of the porcine MDPCs from different passages using Single-cell RNA sequencing (scRNA-seq). Sequencing the RNA of 21 individual mouse satellite cells indicated widespread transcriptional variation (Cho and Doles, 2017). Also, single cell sequencing of mouse muscle stem cells and progenitors helped to precisely define the core cell types of the myogenic differentiation pathway as well as the single cell transcriptional developmental trajectories directing the transition of the stem/progenitor cells from quiescence to proliferation and differentiation (Dell'Orso et al., 2019). My findings from the immunocytochemical analysis in Chapter 3 indicate that the MDPCs contain multiple cell types.

Single cell analysis of porcine MDPCs may provide a clearer picture of the transcriptional changes occurring in these cells during prolonged in vitro cultivation, as well as the various cell types present. Also, such transcriptional changes could be exploited to improve culture conditions that will support proliferation and maintenance of stemness in these cells. Again, such information could be used to identify potential genes and pathways for functional testing in order to improve pig meat production. Furthermore, this transcriptome data can be used to identify representative surface markers for physical separation of subpopulations within porcine MDPCs.
5.2.2 Generating Immortalised MDPC Lines from Pig

Significant progress has been made in developing protocols to isolate and characterise myogenic populations in various species. Unfortunately, the limited proliferative capacity of satellite cells, by cellular senescence and loss of stemness, following long-term cultivation restricts their use for multiple large-scale experiments (Fu et al., 2015, Montarras et al., 2005, Ding et al., 2017, Charville et al., 2015). As reported in other species, the porcine MDPCs displayed a reduction in myogenic potential and finally lost their stemness following prolonged culturing in vitro. Thus, it would be beneficial to develop stable cell lines that can be used as an in vitro platform for identifying and functionally evaluating novel genes implicated in pig meat production. Likewise, such cell lines could potentially serve as a resource for testing therapeutic potential of muscle stem cell.

Cellular senescence is caused primarily by the progressive shortening of telomeres that occurs with each cell division. This is known as replicative senescence, and it is thought to be an irreversible growth arrest caused by DNA damage signalling from critically short telomeres (D'Adda di Fagagna et al., 2003). Furthermore, regardless of telomere length, inadequate in vitro culture conditions do not support long-term proliferation in some cell types (including myoblasts) (Ramirez et al., 2003a). This can lead to accumulating stress and proliferative arrest, which is known as premature growth arrest stress or aberrant signaling-induced senescence (Stadler et al., 2011).

Overexpression of the catalytic subunit of telomerase, human telomerase reverse transcriptase (hTERT), has been shown to prevent senescence in a variety of human somatic cells that do not express this gene endogenously (Bodnar et al., 1998, Wieser et al., 2008). Similarly, overexpression of the cycle regulators, cyclin-dependent kinase 4 (CDK4) or Bmi1 can bypass stasis in many cell types, including myoblasts, while maintaining normal phenotypes (Ramirez et al., 2003b, Zhu et al., 2007b). Several groups have utilised the ectopic expression of hTERT (to overcome replicative senescence) in combination with CDK4 or Bmi1 (to block the growth arrest due to cell-culture
stress) to successfully immortalise myoblasts from normal and diseased human muscle (Robin et al., 2015, Benedetti et al., 2018a, Lathuiliere et al., 2022, Thorley et al., 2016). Future studies could focus on testing a combination of hTERT and various cell cycle regulators to identify a suitable combination that will effectively immortalise porcine MDPCs. Once a suitable combination has been identified, an immortalised porcine MPDC cell line should be generated. The verification of the immortalisation steps should be assayed by growth curve analysis, gene expression studies, measurement of telomere length as well as assessment of telomerase activity. While the combination of hTERT and Bmi-1 resulted in the immortalization of human satellite cells, the immortalised clones failed to differentiate in vitro (Cudré-Mauroux et al., 2003). Also, the combination of hTERT, CDK4R24C and cyclin D1 successfully immortalised human myoblasts but drastically reduced their differentiation capacity in vitro (Chua et al., 2019). Therefore, the in vitro differentiation capacity of the immortalised cells should be tested at various passages. In addition, their ability to regenerate damaged muscle and home in the correct niche in vivo should be tested by injection into an immunocompromised mouse model. Furthermore, karyotype analysis and tumorogenic assays should be done to confirm the immortalised cells maintain normal karyotype and do not form tumors in vivo.

Following the validation of the immortalised myogenic porcine MDPC cell lines, it may be necessary to optimise culture conditions that allow for isolation of clonal porcine MDPCs cell lines. My initial characterisation of these cells showed that they contain a proportion of myogenic and non-myogenic cells. Without clonal MDPC enrichment, testing gene functions in these cells may lead to misleading conclusions as expression of genes differs between different stem cell populations. With respect to developing muscle transfer therapy against muscle myopathies, an ideal cell should have a defined characteristic. Previous research using immortalised myoblasts has shown that mass-infected cultures containing many marked clones contributed disproportionately to muscle regeneration, whereas all clonally isolated lines
survived and regenerated skeletal muscle vivo (Cousins et al., 2004). Therefore, it is essential to isolate clonal cell lines for downstream applications.

5.2.3 Investigating Different Ages / Sex

In this thesis, I have demonstrated a simple and efficient to isolate myogenic cells from porcine skeletal muscle and show the in vitro characteristics of these cells during prolonged culturing. In the future, more comprehensive analysis should be performed that includes simultaneously samples from males and females as well as samples from different ages because the donor’s conditions influence the quantity of isolated muscle stem cells. The potential benefits of studying different ages include the ability to identify age-related changes in biological processes and diseases as well as identifying potential biomarkers for age-related diseases, which can aid in earlier diagnosis and treatment.

Firstly, age-related declines in satellite cell number were reported in studies analysing the number of SCs obtained from young and aged humans and animals including pigs and rodents (Manzano et al., 2011). For example, it has been reported that the number of satellite cells decreased in adult when compared to neonatal mice muscle (Gibson and Schultz, 1983) and pig muscle (Ding et al., 2017). Also following an examination of nine different types of muscle tissues in the pig, including the semimembranosus, semitendinosus, biceps femoris, peroneus tertius, intercostal, longissimus thoracis, psoas major, rhomboideus, and extensor carpi radialis, the highest number of SCs were detected in the psoas major and extensor carpi radialis (Ding et al., 2017).

With respect to in vitro fusion capacity of isolated SCs, studies have shown that the extent of satellite cell fusion in vitro is both species and age dependent. For example, Brandt et al. (2018) showed that satellite cell isolated from young calves (30 days old) exhibit 60% fusion in vitro compared to roughly 40% fusion in adult isolates, indicating a loss in fusion capacity with age (Ge et al., 2012). In the pig, SCs isolated from newborn piglets (5 days old) or juvenile
pigs (5 to 6 weeks old) generate large multinucleated fibre with a fusion index of about 60%. In comparison, only about 30% of adult pig satellite cells fuse into myotubes when cultured in differentiation media in vitro (Zhu et al., 2013, Chen et al., 2017).

Secondly, as sex hormones such as estrogen and testosterone influence the growth of muscle stem cells, sexual dimorphism is another component involved in muscle stem cell growth. It has been shown that sex hormones trigger Mind bomb 1 expression in myofibres and stimulates the Nodal pathway thereby playing a crucial role in establishing the quiescent stem cell population (Kim et al., 2016). In a mouse study, Day et al. (2010) showed that the SC number per myofiber was higher in males compared to females and that males showed a more dramatic reduction in SC number with age compared to females. Moreover, while quiescent SCs were present at a similar rate between adult male and female mice, proliferating SCs were found to be more prevalent in males than females (Neal et al., 2012).

Studies unravelling sex differences in satellite cell number and behaviour in vitro in pig are limited. Overall, investigating different ages and sexes during long term culturing of MDPCs in vitro in the future can help provide a more comprehensive understanding of biological processes in muscle growth and development and specifically, SC behaviour in an agricultural relevant species like the pig.

5.3 Limitations of this Thesis

Some limitations in the studies detailed in this thesis have already been mentioned in previous sections. Here I highlight the major ones.

Samples from >3 animals should have been ideally analysed in my studies. Furthermore, the piglets used in this study came from different litters and herds, which could introduce genetic variations. A larger sample size might enable some of the observed differences to reach statistical significance.
Pericytes believed to be in vivo counterparts of MSCs have been isolated from human and tissues including skeletal muscle based on the expression of CD146 (Crisan et al., 2008b). These pericytes have been shown to maintain typical MSC phenotype in vitro. In chapter two, the in vitro differentiation potential of the CD146+ cells was only tested for myogenic and adipogenic lineages. Therefore, whether these cells are true pericytes capable of trilineage differentiation is not known. Future studies should test if those porcine muscle CD146+ cells meet the minimal criteria required to define MSCs (Dominici et al., 2006). These characteristics include the ability to adhere to uncoated tissue-culture dishes, express MSC markers such as CD105, CD73, and CD90 while lacking expression of CD45, CD14/11b, CD79/CD19, and HLA-DR (Human Leukocyte Antigen - DR isotype) surface molecules, and undergo trilineage differentiation (adipogenesis, chondrogenesis and osteogenesis). Furthermore, it is unknown if the CD146+ cells are capable of contributing to angiogenesis or muscle regeneration in vivo. This information is crucial for understanding the therapeutic potential of these cells and should be tested in future studies by transplanting them into cardiotoxin-induced muscle injury in immunodeficient recipient mice and performing the chorioallantoic membrane angiogenesis assay (Esteves et al., 2017, Ding et al., 2017).

In chapter three, the myogenic potential of the porcine MDPCs was only tested in vitro. While this gives an indication of the lineage plasticity of these cells, whether these cells are capable of regenerating damaged muscle in vivo remains unknown and should be investigated in future studies. Besides, the myogenic and adipogenic lineages, other lineages such as osteogenesis and fibrogenesis should be tested to provide a better picture of the robust nature of the porcine MDPCs and provide further information for their future utilisation.

Due to time constraints, CRISPR/Cas9-mediated gene KO was conducted in MDPCs from only one animal (Chapter 4). Future studies should test the feasibility of this approach in cells from additional animals and include appropriate controls, such as an additional vector-only control, to rule out the possibility that the effects on adipogenesis seen here were caused by the
presence of the Cas9 vector itself. However, the results of the siRNA-mediated knockdown were able to validate the CRISPR/Cas9 data as the knockdown and knock out of KLB produced comparable outcomes. Also, although KLB deletions were confirmed in the single clonal lines, the effect of KLB deletion on adipogenesis was not tested in these clones due to shortage of time. Therefore, it is not known if the observations reported for the mixed pool also hold true for the clonal lines. In future studies, individual clones should also be tested.

5.4 Conclusion

The purpose of the work described in this thesis was to develop and characterise an in vitro porcine skeletal muscle model to be used towards meaningful interrogation of candidate genes involved in muscle growth and development in livestock and, potentially, in vitro meat applications in the future. The results confirm previous findings in other species that muscle tissue is a source of multipotent progenitor cells that express a variety of markers and have varying lineage plasticities in vitro. First, my results demonstrate that both myogenic and adipogenic precursor cell populations can be isolated from pig skeletal muscle using CD146 surface marker, and that CD146+ cells are distinctly myogenic in vitro. These findings extend previous observations in humans and provides an additional marker panels that can be used to characterise porcine myogenic progenitors. Secondly, my results describe a simple and robust explant culture-based approach to obtain and generate readily expandable myogenic and adipogenic precursor cell populations in vitro. By providing a detailed characterisation of the dynamics of this model during prolonged in vitro culturing, this study contributes to the understanding of porcine muscle stem cell behaviour. Lastly, through gain and loss of function studies in the porcine MDPCs I demonstrated the usefulness of the developed model for functional gene studies on pig muscle by unravelling important functions of FGF21-KLB signalling in promoting skeletal muscle fibro/adipogenesis.
Overall, my results provide a strong foundation for further elucidation of the molecular mechanisms involved in normal and abnormal muscle function in pig which in the long-term will benefit the efficiency of meat production. In addition, the porcine muscle stem cell model developed in my studies could prove useful in the development of cell-based therapies by serving as a model for human medical applications. Finally, this cell system could also benefit the production of in vitro cultured meat for human consumption.
References


ADAMS, A. C., YANG, C., COSKUN, T., CHENG, C. C., GIMENO, R. E., LUO, Y. & KHARITONENKOV, A. 2013. The breadth of FGF21's metabolic actions are governed by FGFR1 in adipose tissue. *Molecular Metabolism, 2*, 31-37.


BACHMAN, J. F., KLOSE, A., LIU, W., PARIS, N. D., BLANC, R. S., SCHMALZ, M., KNAPP, E. & CHAKKALAKAL, J. V. 2018. Prepubertal skeletal muscle growth requires Pax7-


HAUSMAN, G. 2012. Adipogenesis in fetal pig subcutaneous adipose tissue: remarkable developmental features before the onset of adipogenesis.


KARUNARATNE, J., ASHTON, C. & STICKLAND, N. C. Prenatal undernutrition increases fat deposition and collagen content within skeletal muscle in the porcine fetus.


KLIMCZAK, A., KOZŁOWSKA, U. & KURPISZ, M. 2018. Muscle stem/progenitor cells and mesenchymal stem cells of bone marrow origin for skeletal muscle regeneration in


LAPAN, A. D. 2012. Melanoma Cell Adhesion Molecule is Associated with Myogenicity in Multiple Progenitor Populations within Human Fetal Skeletal Muscle. Harvard University.


MENG, J., MUNTONI, F. & MORGAN, J. 2018. CD133+ cells derived from skeletal muscles of Duchenne muscular dystrophy patients have a compromised myogenic and muscle regenerative capability. Stem Cell Research, 30, 43-52.


SAMPADOLESI, M., TORRENTE, Y., INNOCENZI, A., TONLORENZI, R., D’ANTONA, G., PELLEGRINO, M. A., BARRESI, R., BRESOLIN, N., DE ANGELIS, M. G. C. & CAMPBELL,


TORRENTE, Y., BELICCHI, M., SAMPAOLESI, M., PISATI, F., MEREGLALLI, M., D’ANTONA, G.,
circulating AC133+ stem cells restore dystrophin expression and ameliorate function
TORRENTE, Y., TREMBLAY, J.-P., PISATI, F., BELICCHI, M., ROSSI, B., SIRONI, M., FORTUNATO,
F., EL FAHIME, M., D’ANGELO, M. G. & CARON, N. J. 2001. Intraarterial injection of
muscle-derived CD34+ Sca-1+ stem cells restores dystrophin in mdx mice. The
Journal of cell biology, 152, 335-348.
TOTLAND, G. K., KRYVI, H. & SLINDE, E. 1988. Composition of muscle fibre types and
connective tissue in bovine M. semitendinosus and its relation to tenderness. Meat
science, 23, 303-315.
Mesenchymal progenitors distinct from satellite cells contribute to ectopic fat cell
formation in skeletal muscle. Nature Cell Biology, 12, 143-152.
UEZUMI, A., FUKADA, S., YAMAMOTO, N., NAKATANI, M., MORITA,
and characterization of PDG-FRalpha+ mesenchymal progenitors in human skeletal
UEZUMI, A., FUKADA, S., YAMAMOTO, N., IKEMOTO-UEZUMI, M., NAKATANI, M., MORITA,
Identification and characterization of PDGFRα+ mesenchymal progenitors in human skeletal
UEZUMI, A., IKEMOTO-UEZUMI, M., ZHOU, H., KUROSAWA, T., YOSHIMOTO, Y., NAKATANI,
Bmp3b expression maintains skeletal muscle integrity and decreases in age-related sarcopenia.
The Journal of clinical investigation, 131.
UEZUMI, A., OJIMA, K., FUKADA, S.-I., IKEMOTO, M., MASUDA, S., MIYAGO-E-SUZUKI, Y. &
TAKEDA, S. I. 2006. Functional heterogeneity of side population cells in skeletal
muscle. Biochemical and biophysical research communications, 341, 864-873.
and environmental aspects of litter size in swine. Journal of Animal Science, 25, 1148-
1153.
of adult porcine mesenchymal stem cells induced by prolonged passaging in culture.
Journal of cellular physiology, 205, 194-201.
VAN DEN BRINK, S. C., SAGE, F., VÉRTESY, Á., SPANJAARD, B., PETERSON-MADURO, J.,
reveals dissociation-induced gene expression in tissue subpopulations. Nature
methods, 14, 935-936.
Transcriptional profiling of quiescent muscle stem cells in vivo. Cell reports, 21, 1994-
2004.
Transcriptional Profiling of Quiescent Muscle Stem Cells In Vivo. Cell Rep, 21, 1994-
2004.
VANDANMAGSAR, B., WARFEL, J. D., WICKS, S. E., GHOSH, S., SALBAUM, J. M., BURK, D.,


Appendixes
Appendix 1 PX458 (pSpCas9(BB)-2A-GFP) Vector
Appendix 2 PX458-mcherry vector