A bovine muscle cell model system is an excellent tool when studying factors important for meat quality

Sissel B. Rønning[1], Mona E. Pedersen[1] , Petter V. Andersen[1] and Kristin Hollung[1]

1 Nofima AS, Pb 210, NO-1431 Ås, Norway.

*sissel.ronning@nofima.no

Abstract – There is an urgent need for cost-effective animal production due to a more competitive meat market. At the same time it is important to maintenance animal welfare in combination with a sustainable meat production. By using cell model systems we can study factors important for eating quality and meat products, without the use of living animals. Understanding the fundamental cellular mechanisms may then help selecting animals for breeding that have a desired muscle growth and produce meat with high quality. We have developed a bovine primary muscle cell model system, using muscle cells from *Longissimus thoracis*. Fresh muscle biopsies were collected at an industrial abattoir, and primary muscle cells were grown and differentiated. A combination of several extracellular matrix components, such as fibrous proteins and glycosaminoglycans, improved the muscle cell growth. The proteoglycan syndecan-4 was important for the fusion of myoblasts into myotubes. Finally, we have studied some of the cellular mechanisms that are involved in post mortem processes.

I. INTRODUCTION

A predictable meat quality is essential for the industry, with tenderness, juiciness, binding properties and shelf-life being the most important quality attributes. These quality traits are controlled by both genetic and environmental factors as well as processing conditions. Selecting animals for breeding, having the optimal genetics for meat quality, depends on knowledge of the molecular mechanisms that determine these quality traits. Studying these factors in live animals are expensive, complicated due to ante-/post- slaughter conditions, genetics, feeding etc., and it is difficult from an ethical point of view. By using muscle cell cultures as a model system diverse factors can be studied in a controlled manner and thus provide us with detailed information on the effect of environmental conditions on muscle cells and muscle tenderization mechanisms. Currently primary model systems from skeletal muscle cells exist for several farm animals, including pig, sheep, and turkey [1-3], as well as in rodents (summarized in [4]).

During skeletal muscle development, muscle cells (myoblasts) proliferate, align with each other, and then fuse to form multinucleated myotubes which then differentiate into muscle fibres. The conversion of myoblasts into multinucleated myotubes is a complex process, and is not fully understood. Muscle growth is governed by its micro-environment, consisting of surrounding cells, vascular system and the extracellular matrix (ECM) (reviewed in [5]). The basic role of the ECM is to provide a supportive scaffold for the cells, promoting cell migration and cell aggregation. The ECM can also directly influence cell behavior through ECM-specific receptors on the cell surface, and indirectly by sequestering and storing soluble growth factors which are then presented at the cell surface at relevant times. The ECM consists of fibrous proteins such as collagens, laminin and entactin [5, 6]. The ECM also contains proteoglycans (PG), which are proteins with long linear sugar chains (GAG chains) attached to the protein core. The composition of the ECM and the structural muscle proteins that make up the muscle fibres are important for the texture of the meat. Understanding the interplay between ECM and growth, and performance on muscle cells can thus provide important information useful for the meat industry.

Post-slaughter conditions will affect the eating quality of beef, and the bovine cell model system is an excellent tool for studying single effects of meat quality parameters. After animal bleeding, muscle tissue enter an anoxic state which impacts all metabolic pathways, and muscle cells develop tools for cell death/survival pathways [7]. Programmed cell death (apoptosis) is believed to be the first step in the conversion of muscle into meat [8], but the dying process and its involvement in meat quality is still not fully characterized.

We have in the present study developed a bovine primary muscle cell *in vitro* model system. We have measured how the ECM influence muscle cell growth and differentiation. Furthermore, we have measured the response to anoxia, simulating post-mortem events.

II. MATERIALS AND METHODS

Cell culture and treatment- Tissue culture coverslips or flasks were coated with Entactin-Collagen IV-Laminin (ECL). Bovine primary skeletal muscle cells were isolated and cultivated essentially as described [9-11]. All experiments were performed in 2nd or 3rd passage. *RNA isolation and real-time PCR* - Cell cultures were purified by RNeasy mini kit including a DNase treatment. cDNA was generated from ~200 ng mRNA using TaqMan® Reverse Transcription Reagents. Gene expression of the samples was normalized against TATA, and ΔCt was calculated, according to the MIQE guidelines [12]. *Immunocytochemistry and fluorescence microscopy*- Cells were grown on coated coverslips, fixed in 2 % PFA for 15 min before immunostaining. The cells were examined by fluorescence microscopy analysis (apotome mode) (ZEISS Axio Observer Z1 microscope, Jena, Germany). *Western blot*- Proliferating and differentiating cell cultures were lysed and subjected to SDS-Page gel electrophoresis and western blotting.

III. RESULTS AND DISCUSSION

Bovine primary skeletal muscle cells isolated from *Longissumus thoracis* demonstrated after five days a typical morphology (triangle shaped), growing as an even layer of single cells (Fig. 1A). The muscle cells underwent proliferation with a doubling time of approximately 2.2 days (Fig. 1B). When the cells reached a density of 80-90% confluence, the cells started to fuse into multinucleated myotubes (Fig. 1C). We investigated the protein level of MyoD, and Desmin in proliferating cells (day 0) and differentiating cells (day 3) (Fig. 1D). The expression of MyoD persisted during the differentiation. The protein expression of Desmin, on the other hand, increased significantly after three days.

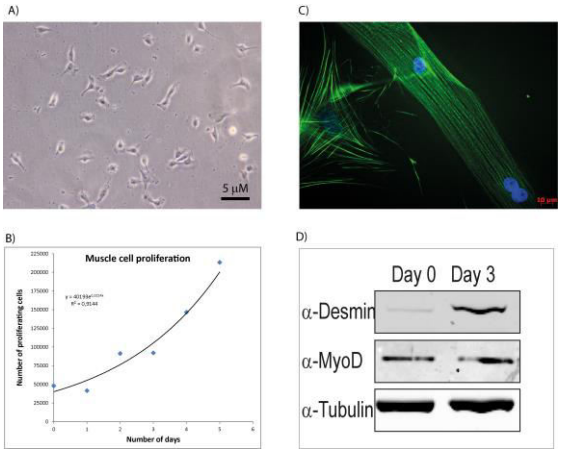

Fig.1: A) Cell growth five days after harvest. B) Quantification of cell growth. C) Differentiated cells were stained for actin (green) before fluorescence microscopy. Nuclei were stained with DAPI (blue). D) A representative western blot showing the expression level of MyoD and Desmin after Day 0 and Day 3. Cell lysates were subjected to western blotting using antibodies to MyoD, Desmin, and Tubulin (loading control).

To investigate the effects ECM proteins have on cell growth and differentiation, we cultured the isolated cells on various surfaces coated either with collagen alone or a complex ECM surface coating (ECL+GAGs).

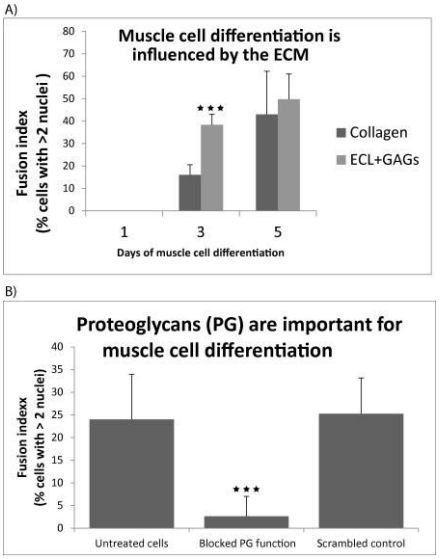

Fig. 2: A) Surface coatings influence the cell differentiation. B) Blocked PG function inhibits muscle differentiation.

The muscle cells cultured on ECL+GAGs clearly differentiated faster compared to cells cultured on single protein coatings (Fig. 2A). Furthermore, when blocking the function of the PG syndecan-4 a dramatic reduction in differentiation was observed (Fig. 2B).

Removing oxygen (anoxia) lead to an increase in mRNA expression of anti- and pro apoptotic markers (Fig. 3A), while the cells were still metabolically active (Fig. 3B). Mitochondria play a central role in apoptosis, and is probably involved in development of meat qualities [13]. A loss of mitochondrial membrane potential was observed during anoxia (Fig. 3C, right panel).

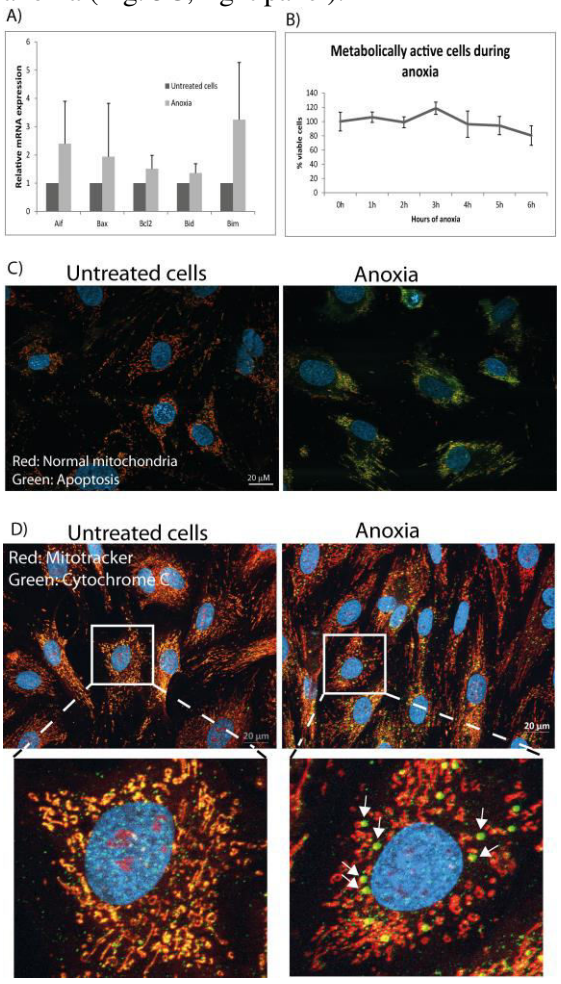

Fig. 3: A) Relative mRNA expression of apoptosis markers increase upon oxygen removal. B) Viable cells during anoxia based on quantitation of ATP present. C) Live fluorescence microscopy of cells during anoxia in combination stained with an apoptosis marker (red: normal/green: apoptosis). D) Fluorescence microscopy of cells during anoxia, stained with Mitotracker (red) and CytC (green). Arrows = release of CytC from mitochondria in cytosol. Nuclei were stained with DAPI (blue).

Finally, a release of cytochrome C from the mitochondria to the cytosol was observed (Fig. 3D, right panel). The release of cytochrome C leads to the formation of the apoptosome, and this complex is essential for caspase activation. Cytochrome C may also act as an intracellular intermediate that deregulates the sarcoplasmic reticulum Ca^{2+} flux [13].

IV. CONCLUSION

In conclusion, using primary cell model systems, it is possible to study different factors, both ante- and post-slaughter conditions, which influence eating quality of beef. We have demonstrated how important the ECM is for muscle growth and development. Furthermore, mimicking slaughter conditions by removing oxygen, show that apoptosis is induced as an early post-mortem event. The model system provides an excellent tool that can be used to study muscle growth, how nutrients affect muscle growth, the commutation between the ECM and muscle cells, and post-mortem factors important for meat tenderization. This will eventually help extending our knowledge regarding molecular mechanisms important for high meat quality, and to ensure a consistent meat production.

ACKNOWLEDGEMENTS

We thank Grethe Enersen for technical support. This work was supported by grants from the Fund for Research Levy on Agricultural Products in Norway, the Norwegian Research Council (203697/O99).

REFERENCES

1. Wilschut KJ, Haagsman HP, Roelen BAJ. (2010) Extracellular matrix components direct porcine muscle stem cell behavior. Experimental Cell Research 316:341-52.
2. Wu H, Ren Y, Li S, Wang W, Yuan J, Cang M, et al. (2012) In vitro culture and induced differentiation of sheep skeletal muscle satellite cells. Cell Biol Int.
3. Velleman SG, Liu X, Nestor KE, McFarland DC. (2000) Heterogeneity in growth and differentiation characteristics in male and female satellite cells isolated from turkey lines with different growth rates. Comparative biochemistry and physiology Part A, Molecular & integrative physiology 125:503-9.
4. Yablonka-Reuveni Z, Day K. (2011). Skeletal Muscle Stem Cells in the Spotlight: The Satellite Cell. Regenerating the Heart. In: Cohen I, Gaudette G, editor.^editors.: Humana Press; p. 173-200.
5. Thorsteinsdottir S, Deries M, Cachaco AS, Bajanca F. (2011) The extracellular matrix dimension of skeletal muscle development. Developmental Biology 354:191-207.
6. Frantz C, Stewart KM, Weaver VM. (2010) The extracellular matrix at a glance. Journal of cell science 123:4195-200.
7. Ouali A, Gagaoua M, Boudida Y, Becila S, Boudjellal A, Herrera-Mendez CH, et al. (2013) Biomarkers of meat tenderness: present knowledge and perspectives in regards to our current understanding of the mechanisms involved. Meat Sci 95:854-70.
8. Becila S, Herrera-Mendez CH, Coulis G, Labas R, Astruc T, Picard B, et al. (2010) Postmortem muscle cells die through apoptosis. Eur Food Res Technol 231:485-93.
9. Gaster M, Beck-Nielsen H, Schroder HD. (2001) Proliferation conditions for human satellite cells. The fractional content of satellite cells. APMIS 109:726-34.
10. Gaster M, Kristensen SR, Beck-Nielsen H, Schroder HD. (2001) A cellular model system of differentiated human myotubes. APMIS 109:735-44.
11. Rønning SB, Pedersen ME, Andersen PV, Hollung K. (2013) The combination of glycosaminoglycans and fibrous proteins improves cell proliferation and early differentiation of bovine primary skeletal muscle cells. Differentiation 86:13-22.
12. Bustin SA, Beaulieu JF, Huggett J, Jaggi R, Kibenge FS, Olsvik PA, et al. (2010) MIQE precis: Practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. BMC molecular biology 11:74.
13. Sierra V, Olivan M. (2013) Role of mitochondria on muscle cell death and meat tenderization. Recent patents on endocrine, metabolic & immune drug discovery 7:120-9.