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REPLY TO LETTER TO THE EDITOR

Reply to the Issue of Skeletal Muscle Growth and Regeneration

Peter MAKOVICKÝ[1], Pavol MAKOVICKÝ[2]

[1]Czech Centre for Phenogenomics, Institute of Molecular Genetic of the Czech Academy of Sciences, Vestec, Czech Republic. [2]Department of Biology, Faculty of Education, J. Selye University, Komarno, Slovak Republic

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Corresponding author

Peter Makovický, Czech Centre for Phenogenomics, Institute of Molecular Genetic of the Czech Academy of Sciences, Průmyslová 595, 252 50 Vestec, Czech Republic. E-mail: peter.makovicky@img.cas.cz

Here, we would like to provide a short response to Letter to the Editor written by a respected human anatomists and histologists (Varga *et al.* 2020). The muscles of animals represent 40 to 50 % of their body weight, and the greatest part of this mass consists of cross-striated skeletal muscles (Makovicky *et al.* 2009d). At present it is a subject of intensive veterinary research, as muscles represent one of the most important elements in human nutrition. In addition, they are at the centre of applied research especially for muscular dystrophies, which are histologically characterized by muscular fiber atrophy, fragmentation and necrosis. Therefore, muscular fiber regeneration is an important part of research, but reparation is only the final stage of skeletal muscle damage. It seems that gene mutations play major roles in muscular dystrophies. On the other hand, it can also be expressed that myopathology in veterinary medicine is still lacking sufficient knowledge, but we are certain that future discoveries about individual gene mutations will change the view of skeletal muscle disease classification. Several studies have concentrated on the pathophysiology of muscular dystrophies, and muscular fiber splitting is interpreted here as a degenerative process. From this, several experimental studies documented muscular fiber splitting as a result of intensive exercise (Antonio and Gonyea 1994, Ho *et al.* 1980, Sola *et al.* 1973) or also exercise and nutrition (Eriksson *et al.* 2006). Some authors are convinced that this process starts with skeletal muscle fiber hyperplasia and is followed by skeletal muscle fiber hypertrophy (Alway *et al.* 1989, Tamaki *et al.* 1992). One study documented muscle fiber splitting in the normal skeletal muscle of dogs without disease (Braund *et al.* 1982), or one necroptic study documented muscle fiber splitting in the skeletal muscles of horses (Valentine 2008). Other authors are convinced that muscular fiber can grow only to a limited thickness after they have split (Uhrín and Uhrín 1989). It was even proposed that mice are not the best animal species for potential muscular dystrophy therapeutic studies because results obtained in mice cannot always by replicated in humans (Duan *et al.* 2015). We are supposing that skeletal muscle fiber splitting can also be visualized in C57BL/6NCrl mice. Today, there are models for several phenotyping centers across the world. Taking into account that skeletal muscles are constantly sampled during phenotyping, we hold the opinion that it is important to monitor and report if there are some changes in skeletal muscle.

Muscle spindles are a persistent part of animal skeletal muscles, functioning as sensory receptors and playing an important role in muscle contraction. For example, there was a full presentation about skeletal muscle development at the International Congress of Slovak Anatomical Society and 44th Lojda Symposium on Histochemistry, held in Bratislava. During the presentation, muscle spindles were also mentioned (Makovický *et al.* 2007d). In another International Congress on Anatomy and 49th Lojda Symposium on Histochemistry, held in Hradec Kralove, there was a full presentation showing the histological aspects of muscular

Fax +420 241 062 164, e-mail: physres@fgu.cas.cz, www.biomed.cas.cz/physiolres

fiber splitting, containing several detailed figures (Makovický *et al.* 2012). There have been several our presentations and/or articles dealing with the development of animal skeletal muscle (Kulíšek *et al.* 2004a, Kulíšek *et al.* 2004b, Makovický *et al.* 2007c, Makovický *et al.* 2009c), including the role of satellite cells or the impact of the *MYF4* gene during skeletal muscle development (Makovický *et al.* 2006, Makovický *et al.* 2009a, Makovický *et al.* 2015). Special attention was given to giant fibers and muscular fiber splitting (Makovický *et al.* 2007a, Makovický and Makovický 2015), with several histological changes observed, including the influence of the *RYR1* gene and histological, histochemical changes in muscular fibers (Makovický *et al.* 2007b). A classification system for the splitting muscle fibers in the form of histological grade (G1-G4) was proposed and recommended for veterinary biopsy practice (Makovický *et al.* 2009b). Morphometric analyses were made and some histochemical properties of split skeletal muscles were identified (Makovický *et al.* 2010).

All the samples are derived from our IMPC (International Mouse Phenotyping Consortium) phenotyped mice. General information about the program is freely available. The consortium began work in 2011 with its first and immediate objective to generate a null mutant and undertake broad based phenotyping for every gene in the mouse genome. As of 2018, IMPC have completed 8,000 genes – more than a third of the mouse coding genome. Completing the null mutant resource will enable to define the complete catalogue of essential genes in the mammalian genome, as well as continue to elaborate and expand the view of the genome landscape for multiple disease areas. The phenotyping module at the Czech Center for Phenogenomics houses a comprehensive collection of tools for the physiological and morphological assessment of experimental mice and rats in a controlled SPF (specific pathogen-free) environment. All animal models and experiments used in study were ethically reviewed and performed in accordance with European directive 2010/63/EU and were approved by the Czech Central Commission for Animal Welfare. Each cohort contains prescribed mice, and from selected mice 30 female and 32 male organs are sampled, including two cytological smears. Our database currently contains 1219 (629 females; 590 males) C57BL/6NCrl mice. This study reflected our personal views and shows a selected portion of our results about skeletal muscles, but could prove to be useful for the highly focused community in rodent histopathology. Results document normal skeletal muscles, and other macroscopic, histological results will be applied to particular mutant cohort mice, or to full control mice, and may be published in a future article. From the 500 samples discussed, those that possess split muscle fibers make up less than 1 %, which on calculation is a negligible amount. If we were to compare this with the amount of muscle spindles in skeletal muscle, it is clear that there is a difference in their numbers, which would lead to a result of more than 1 %.

Table 1. Percentage average of mice with hypertrophic/splitting muscular fibers and percentage average of hypertrophic/splitting muscular fibers on each individual sample.

Signature	MHSMF		AHSMF	
	250 ♀	250 ♂	FSM ♀	FSM ♂
HMF	<1%	<1%	<1%	<1%
SMF	<1%	<1%	<1%	<1%

MHSMF: percentage average of mice with hypertrophic and splitting muscular fibers, AHSMF: percentage average of hypertrophic and splitting muscular fibers on each individual sample, FSM: femoral skeletal muscles, HMF: percentages average of hypertrophic muscular fibers, SMF: percentages average of splitting muscular fibers.

For analysis, slides with hypertrophic or splitting muscular fibers were scanned using a Carl Zeiss Axio Scope A1 (Zeiss, Germany), which allows morphometry analysis (Kučera 2018). The average percentage of hypertrophic and splitting muscular fibers was calculated. An independent reviewer recommended that we remove a table containing morphometric results from our paper (Makovický and Makovický 2020). Table 1 shows that hypertrophic and splitting muscular fibers are visible in less than 1 % of all mice and less than 1 % of each individual sample. Muscular spindles are also clearly visible in the skeletal muscle of C57BL/6NCrl Mice. We acknowledge the figure criticism, which correctly points to the presence of muscle spindles. It was an oversight not to mention muscular spindles in our results section, nor in our figure descriptions. However, it shall not be overlooked in our future works. It seems that splitting muscular fibers have different morphology during pathological conditions compared to spontaneous splitting. Even in normal muscles they are only occasionally visible, in one mutant cohort of mice we found an increasing number of split muscular fibers.

In our work, we do not discuss large-scale skeletal muscle regeneration or skeletal muscle

degeneration, muscular fibers fragmentation and necrosis, which is accompanied by changes in the muscular fiber shape, color and fibrosis (Fig. 1A). What we do discuss is spontaneous muscular fiber splitting. Usually there is one hypertrophic muscular fiber (Fig. 1C), which undergoes splitting, but also one miniature splitting muscle fiber that is occasionally visible (Fig. 1D). Alternatively, the finding resembling an individual regenerating muscle fiber, which are histologically characterized by the presence of prominent cells, rows of internal myoblast nuclei and cytoplasmic basophilia (Nonneoplastic Lesion Atlas 2014). Figure 1E documents a row of internal vesiculated nuclei in regenerating muscle fibers. In accordance to Nonneoplastic Lesion Atlas (2014), which is a guide for standardizing terminology in toxicological pathology for rodents, they tend to retain their internal nuclei for some time, and this can serve as a marker for fibers that had previously undergone regeneration. Figure 1B documents muscular fibers with centrally localized nuclei. We are aware that the full view can be influenced by sampling and processing. Despite this, we believe that splitting muscle fiber are visible also near muscular spindles (Fig. 1F). This is our personal opinion, and we hope that our future work will shed more light on this issue. All the sampling materials, including sampling strategy, etc. strictly need to follow protocol. If we would like to make changes, it is only possible with additionally sampled material. Formol-parafin techniques in skeletal muscle histology often cause several artifacts and in order to minimize them, serial sections are requested. Our previous studies, and other external studies, dealing with skeletal muscles have utilized another fixative and alternative procedures, including additional staining for skeletal muscle evaluation. Certain changes in skeletal muscle fixation, sample processing and additional staining in mice phenotyping have been suggested several times by us. If clinics show signs of a musculoskeletal system disorder, or there are suspected changes in skeletal muscles, sampling from several muscle groups are always suggested. In addition, transversally oriented sections are recommended, so that the muscle fibers are visible in cross-section.

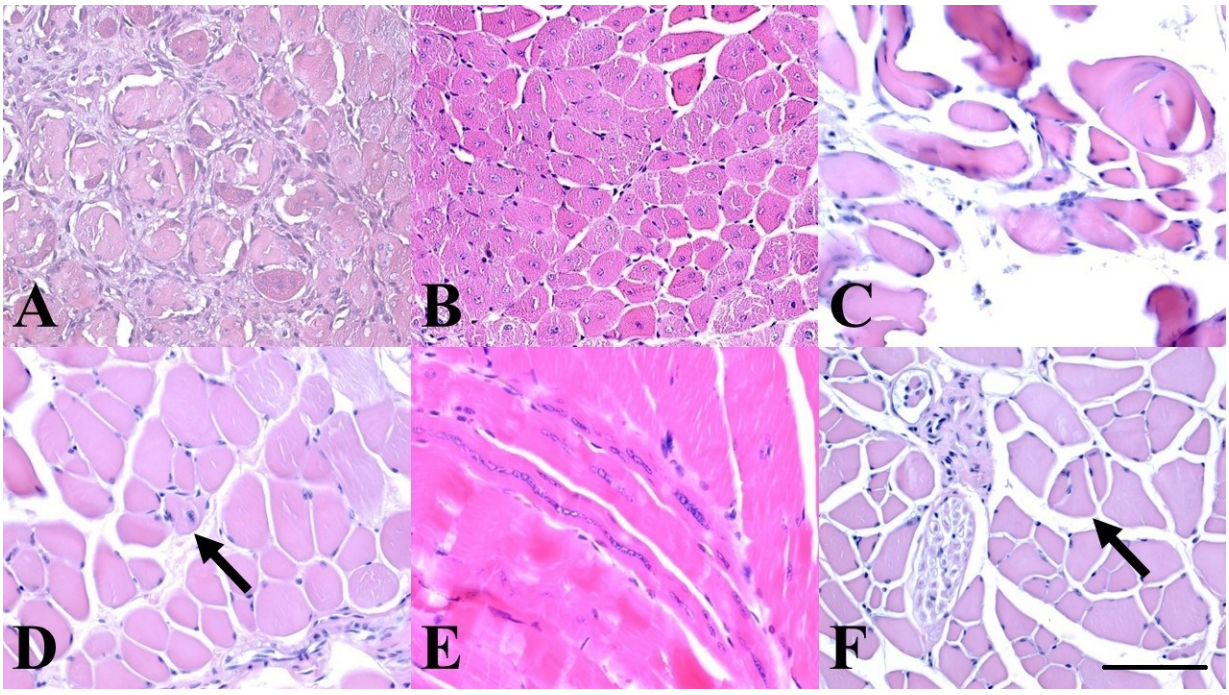

Fig. 1. Selected histological figures with description in the text. Legend: A, B, C, D, E, F: HE: 400x, scale bar: 50 μm.

It is a generally accepted opinion that a set of histochemical and immunohistochemical stainings will distinguish particular cells and tissues within samples and to increase the accuracy of final diagnoses (Makovický and Švecová 2016). Today in Veterinary Pathology, most diagnoses are completed from hematoxylin-eosin staining (Makovický 2015). Although our laboratory has a wider spectrum of additional special staining, it is possible to perform this appropriate staining only on a limited set of skeletal muscle samples. In our future work, a smaller sample number will be described with the appropriate additional staining. This approach is ongoing, especially

in individual external studies. Myology is a separate field which requires its own laboratory and staff. Worldwide, there are several Veterinary Pathology laboratories with varying facilities. Most histological consultations or second reading are realized from hematoxylin-eosin staining. Here, it is not surprising that we can encounter many different interpretations on the same sample. Sample turnover requirements for evaluation exceed the time interval to receive results even in common veterinary biopsy practice.

Finally, we would like to thank Varga and coworkers for their Letter to the Editor, as we do also appreciate criticism. Everyone who shows a serious interest is welcomed to do so, and we are ready to cooperate within our capabilities to consult our findings. The value of our results has not decreased. It is only another view that has raised several questions, which can be expanded in future works concerning muscular fiber splitting. The common objective of research is to discuss the relevant topic and express different views of a common problem. In this way, cooperation between several specialists will be imperative to bring excellent results in the future, not only in skeletal muscle research.

References

ALWAY SE, WINCHESTER PK, DAVIS ME, GONYEA WJ: Regionalised adaptations and muscle fiber proliferation in stretch-induced enlargement. J Appl Physiol 66: 771-781, 1989.

ANTONIO J. GONYEA WJ: Muscle fiber splitting in stretch-enlarged avian muscle. Med Sci Sports Exerc 26: 973-977, 1994.

BRAUND KG, MCGUIRE JA, LINCOLN CE: Observation on normal skeletal muscle of mature dogs: a cytochemical, histochemical, and morphometric study. Vet Pathol 19: 577-595, 1982.

HO KW, ROY RR, TWEEDLE CD, HEUSNER WW, VAN HUSS WD, CARROW RE: Skeletal muscle fiber splitting with weight-lifting exercise in rats. Am J Anat 157: 433-440, 1980.

DUAN D: Duchenne muscular dystrophy gene therapy in the canine model. Hum Gene Ther Clin Dev 26: 57-69, 2015.

ERIKSSON A, LINDSTROM M, CARLSSON L, THORNELL LE: Hypertrophic muscle fibres with fissures in power-lifters: fiber splitting or defect regeneration? Histochem Cell Biol 26: 409-417, 2006.

KUČERA J: Slide scanning technology. Phenogenomic Newsletter 2: 15-17, 2018.

KULÍŠEK V, MAKOVICKÝ P, VAVRIŠÍNOVÁ K: Some aspects of prenatal and postnatal growth of striated muscles of domestic animals. In: Book of Abstracts from International Scientific Seminar: Topical task solved in agro-food sector. Nitra. 65-66, 2004a.

KULÍŠEK V, MAKOVICKÝ P, MLYNEK J, BOBČEK B: Influences on the growth and development of cross striated skeletal muscles. (In Slovak) In: Sborník příspěvků: Aktuální otázky produkce jatečných zvířat. Brno, 32-36, 2004b.

MAKOVICKÝ P: Histological study of giant fibres in skeletal muscles of pigs. Fleischwirt Int 25: 966-968, 2010.

MAKOVICKÝ P: What does modern veterinary pathology have to offer? J Anim Vet Sci 1: 43-47, 2015.

MAKOVICKÝ P, MAKOVICKÝ P: Is postnatal growth of skeletal muscle in pigs performed by muscle fiber cleaving? (In Slovak) Veterinářství 65: 709-717, 2015.

MAKOVICKÝ P, MAKOVICKÝ P: Histological aspects of skeletal muscle fibers splitting of C57BL/6NCrl mice. Physiol Res 69: 291-296, 2020.

MAKOVICKÝ P, ŠVECOVÁ I: Veterinary pathology: the past, present and the future. Phenogenomic Newsletter 2: 22-23, 2016.

MAKOVICKÝ P, KULÍŠEK V, MLYNEK J, HAŠČÍK P: Influence of the transverse striations of skeletal tissues on the growth of farm animals (review). Slov J Anim Sci 39: 218-225, 2006.

MAKOVICKÝ P, MAKOVICKÝ P, KULÍŠEK V, DEBRECÉNI O, HAŠČÍK P: Histological analysis of size, structure and appearance of giant fibres in skeletal muscles of pigs. Folia Vet 51: 5-8, 2007a.

MAKOVICKÝ P, MAKOVICKÝ P, LEVKUT M, CHRENKOVÁ M, KULÍŠEK V, JÍLEK F: Histopathological and genetic aspects of stress syndrome of the skeleton musculature of pigs with resulting PSE meat. Folia Vet 51: 3-4, 2007b.

MAKOVICKÝ P, MAKOVICKÝ P, KULÍŠEK V: Effects of Genetic Factors on Genesis and Growth of Striated Skeletal Muscular Tissues. (In Slovak) Cesk fyziol 56: 15-21, 2007c.

MAKOVICKÝ P, MAKOVICKÝ P, KULÍŠEK V, ŠŤASTNÝ P, BUČKO O: The development and histogenesis of the striated skeletal muscles of pigs. In: Programme and Abstract Book of the 41 st International Congress of Slovak Anatomical Society and 44th Lojda Symposium on Histochemistry: MORPHOLOGY 2007. 65, 2007d

MAKOVICKÝ P, MAKOVICKÝ P, KAČÁNIOVÁ M, PAVLIČOVÁ S, HAŠČÍK P: Histochemical analysis of skeletal muscular tissues of pigs according to genotype MYF 4. Arch Tier/Arch Anim Breed 52: 395-401, 2009a.

MAKOVICKÝ P, MAKOVICKÝ P, CHRENKOVÁ M: Histopathological aspects of giant fibres in selected muscles of the pig. Isr J Vet Med 64: 31-35, 2009b.

MAKOVICKÝ P, MAKOVICKÝ P, LEVKUT M, LEVKUT M, VRTKOVÁ I: Histological and morphometrical parameters of the skeletal muscle development in pigs. Sci Agric Bohem 40: 121-129, 2009c.

MAKOVICKÝ P, MAKOVICKÝ P, KULÍŠEK V, KAČÁNIOVÁ M, PAVLIČOVÁ S, HAŠČÍK P: Morphometrical and histochemical study of the cross striated skeletal muscles of pigs. Slov J Anim Sci 42: 174-179, 2009d.

MAKOVICKÝ P, MAKOVICKÝ P, CHRENKOVÁ M, KULÍŠEK V: Our experiences with splitting of muscle fibers. In: Programme and Abstract Book of the MORPHOLOGY 2012 - 47th International Congress on Anatomy and 49th Lojda Symposium on Histochemistry. Hradec Králové, 90, 2012.

MAKOVICKÝ P, MAKOVICKÝ P, LIPPAI R, SZIKSZ E, SAMASCA G: The development and growth of muscular fibers of striated skeletal muscle. (In Hungarian) Magy Allatorvosok 137: 559-567, 2015.

Nonneoplastic Lesion Atlas. 2014 https://ntp.niehs.nih.gov/nnl/index.htm

SOLA OM, CHRISTENSEN DL, MARTIN AW: Hypertrophy and hyperplasia of adult chicken anterior latissimus dorsi muscles following stretch with and without denervation. Exp Neurol 41: 76-100, 1973.

TAMAKI T, UCHIYAMA S, NAKANO S: A weight-lifting exercise model for inducing hypertrophy in the hindlimb muscles of rats. Med Sci Sports Exerc 24: 881-886, 1992.

UHRÍN P, UHRÍN V: Meat production in terms of structural and functional properties of muscles. (In Slovak) Nitra: VUŽV, 82, 1989.

VALENTINE BA: Pathologic findings in equine muscle (excluding polysaccharide storage): a necroptic study. J Vet Diagn Invest 20: 572-579, 2008.

VARGA I, TONAR Z, GHALLAB A, DANISOVIC L, KLEIN M: The issue of skeletal muscle growth and regeneration. Physiol Res 69: 545-548, 2020.