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IN VITRO INVESTIGATION OF GENES AFFECTING MUSCLE DIFFERENTIATION IN EMBRYONIC STEM CELL LINES IN MOUSE MODEL

Doctoral (PhD) Thesis

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1. Objectives

Meat production still has the greatest proportion within animal production. In quality meat production, those breeds having an outstanding trait related with meat quantity or quality have always been preferred. The so-called *culard* (or double muscle) phenotype fascinate breeders as the high meat yield of animals having that phenotype has always been a sought-after trait in animal husbandry. This phenomenon occurs for instance in the *Belgian White-Blue* or in the *Piedmontese* cattle breeds. In the view of quality meat production, this is an outstanding trait, since these animals produce not just more, but better: leaner and tenderer meat. Crossing with *Belgian White-Blue* cattle shows that although the gene is recessive and monofactorial, its effect is apparent even in heterozygotes due to its partial dominance: the meat:bone ratio and meat yield is better than those of the other breed. In animals with a culard phenotype, this trait manifests with others: shortened tubular bones, less deposition of fat and suet and decreased amount of connective tissues between muscles. It is still not clear if the mutation of the gene that causes hypertrophy is responsible for these effects to occur or other factors may also have a role in it.

Myostatin is a regulator protein in normal muscle that inhibits the expression of genes that are active during the differentiation of muscle tissues, therefore, determining the maximum amount of muscle mass typical of that species. If the myostatin gene is mutant, the negative regulating function of the gene does not work. This leads to an increased muscle growth resulting in muscle hypertrophy. The mutation in the myostatin gene is responsible for the development of the culard phenotype.

The so-called *compact* (*Cmpt*) mouse carrying the mutant myostatin gene can be a model that may help to answer the questions. L. Varga and his colleagues at the ABC did the genetic mapping of the mutation, therefore, the genetic background of the mutation was known for us.

Differentiating embryonic stem cells derived from *compact* mouse into muscle in vitro, we aimed to identify the factors that may have a role in the development of this trait.

The embryonic stem (ES) cells are derived from mouse blastocysts. In favourable conditions, these cells divide constantly. If injected into a host embryo, these cells integrate into the inner cell mass, begin to differentiate, and during embryogenesis, they can develop into different cell types. Supplement of the cultivation medium with factors promoting growth and differentiation results in the appearance of differentiated cells. Thus, ES cell lines may serve as *in vitro* models to study the mechanism of cell differentiation, e.g. the initial steps of muscle differentiation. Compared to *in vivo* events, these cells show slower paced differentiation, thus offer a longer period for observation.

In our experimental system, observation of the differentiating muscle cells enables us to study the role of the regulator proteins similar to myostatin in the development of hypertrophy and to answer the questions related to the role of the

myostatin played in the muscle differentiation cascade. Observations made on *Belgian White Blue* muscle tissue culture may help to adapt the results derived from research performed on mouse as an animal model.

The main objectives of the study work outlined in the thesis:

1. Derivation of pluripotent ES cell lines carrying the mutated myostatin and verification of their pluripotency *in vitro* and *in vivo*.
2. Developing derivation methods in order to successfully establish ES cell lines from non-permissive mouse strains.
3. Comparison of gene expression pattern detected in *in vitro* differentiation of ES cell lines carrying the mutated myostatin into skeletal muscle with control ES cell lines not carrying the mutation.
4. Isolation and *in vitro* differentiation of stem cells of muscle origin obtained from myostatin mutant and control animals. Comparison of gene expressions observed during differentiation.
5. Exploration of the mechanism of action of myostatin by in vitro differentiation of ES and stem cells in muscle in vitro. Parallel observation of action of genes playing role in muscle formation.
6. Detailed histological analysis of the *compact* phenotype caused by mutant myostatin to detect morphological alterations in the developed muscle.
7. Isolation and in vitro differentiation of stem cells and primary muscle cell cultures from hypertrophic (*Belgian White Blue*) and control (*Holstein-Friesian*) cattle. Comparison of the results with those of mice.

2. Materials and methods

For experiments, the mice resulted from the crossing of the myostatin mutant (*mstn-/-*) 'compact' strain and of a transgenic strain homozygote for the Egfp gene. The heterozygote F_1 $mstn^{+/-}/Egfp^{+/-}$ generation was intercrossed. The Egfp homozygotes in the progeny were selected for establishing new sublines: the negative variant control line ($mstn^{+/+}/Egfp^{-/-}$) and the myostatin double mutant and Egfp homozygote transgenic generation ($mstn^{-/-}/Egfp^{+/+}$). These strains entered in an inbreeding programme.

Blastocysts obtained from superovulated and naturally mated females were used for derivation of ES cells.

A new ES derivation method was elaborated based on the use of conditioned medium (in details, see *Results*). Using this method, several myostatin mutant and control cell lines have been established. The pluripotency of the newly derived cell lines was thoroughly analysed *in vitro* (karyotype analysis, alkalic-phosphatase activity, SSEA-1 cell surface antigen expression, Oct-4 gene expression) and *in vivo* (chimaera analysis).

To study muscle differentiation, ES cell lines have been differentiated in hanging drops *in vitro*. The embryoid bodies (EBs) formed in hanging drops were differentiated first in suspension and then placed into gelatine-coated dishes for 20 days. Samples were collected regularly, from which mRNA were prepared. The mRNA samples were studied using RT-PCR for the expression of 17 genes chosen according to published results and our results on searching for transcription factor binding sites on the coding region of the myostatin.

Parallel with the in vitro differentiation of the ES cells, stem cells of muscle origin have been isolated and differentiated *in vitro* and primary muscle cultures have been studied both in mouse and cattle. Gene expression analyses performed during differentiation were identical to those done in ES cells.

Detailed morphological and histological analyses were performed in the *compact* strain. Litter and average individual weights, of myostatin mutant and control mice were compared at birth, at weaning and in adults. Weights of several muscles were also measured and compared.

Histological analysis comprised observations in light microscopy (haematoxylin-eosin, methyl-green-pironin, iron-haematoxylin, azan-, and Van Gieson-dyeing, silver impregnation) and TEM.

Tetrazolium-reductase (NADH-TR) dyeing and morphometric analysis was used to identify fibre types.

The Fisher's exact-test and χ^2 test was used for statistical analysis with a confidence interval of 95%.

3. Results

3.1 Derivation of ES cell lines from mstn$^{-/-}$ mutant mouse lines

An ES cell line derivation method based on conditioned medium was elaborated. The composition of the medium (EM) traditionally used for cultivation of ES cells was not altered but the medium was conditioned with the well-characterised R1 ES cells. The medium was collected from the proliferating cells after 24h cultivation on the first and the second day following the passage. When confluency reached 70%, the cells were passaged or the collection of the medium was ceased. The conditioned medium was centrifuged (5 min, 2500 rpm, 4°C), sterile filtered (0.22 μm), and stored at 4°C until use. Since the cells not just excrete growth factors and hormones into the medium but also use up its amino acids and sugars, the medium was not used directly. In addition, at the temperature of cultivation, the antibiotics, the Na-pyruvate, the $NaHCO_3$ and the amino acids decay easily, so their supply is indispensable for successful cultivation. Therefore, the conditioned ES establishing medium (CEM) was prepared as follows: the LIF (Leukaemia Inhibitory Factor) content of the EM was elevated to 2000 U/ml, and the proportion of the FCS (Fetal Calf Serum) was raised to 20v/v%. The supplemented EM was mixed with the collected conditioned medium in a ratio of 1:1.

The traditional ES derivation method was modified according to the following:

- 3.5-day-old blastocysts were not cultivated in microdrops but, right after flushing, they were placed onto a mitomycin C-treated mouse embryonic fibroblast (MEF, obtained from the first passage) in CEM in a 24-well plate. Cultivation medium was changed only after the hatched blastocyst attached to the fibroblast.
- On the 3rd and 4th day of the ICM formation, passage was performed using trypsin and mechanic manipulation onto mitomycin C-treated MEF layer.
- The medium was changed every other day until the appearance of ES-like colonies.
- It took at least 10 days for the colonies to appear.
- Using mechanical manipulation and trypsin, the ES colonies were placed onto a mitomycin C-treated mouse embryonic fibroblast (MEF, obtained from the first passage) in CEM in a 12-well plate. Cultivation medium was changed only after the hatched blastocyst attached to the fibroblast.
- When the number of colonies reached a sufficient level, the cells were passaged (passage № 1) into a 6 cm Petri dish. The first freezing was also performed from these cultures.
- The cell lines were cloned in the 2nd but not later than in the 3rd passage.
- The cell lines were cultivated until the 4th or 5th passage in CEM, then the medium was gradually changed to EM.

Using CEM, 5 myostatin mutant (*mstn*$^{-/-}$*/Egfp*$^{+/+}$) and 2 control (*mstn*$^{+/+}$*/Egfp*$^{-/-}$) cell lines have been established. Out of these, 3 mutant (*mstn*$^{-/-}$*/Egfp*$^{+/+}$*1, mstn*$^{-/-}$*/Egfp*$^{+/+}$*2, mstn*$^{-/-}$*/Egfp*$^{-/-}$*5*) and a control (*mstn*$^{+/+}$*/C7*) lines have been chosen. Their pluripotency was thoroughly tested in vivo and in vitro. When the cell lines were analysed, the myostatin mutation was genotyped again. According to the results, the cell lines proved to be pluripotent.

3.2 Studies on the effects of conditioned medium in other mouse strains

The myostatin mutant mice had 129SV background. Therefore, the results detailed above alone are not sufficient to prove the efficiency of the method and of the medium. Thus, the medium was tested on other strains (F2, CBA, BalbC, and NMRI) as well. The conditioned medium and the modified method of ES derivation were compared with the 'classic' method.

Using the 'classic' method no ES cell line could be produced from any of the five strains (129SV, F2, CBA, BalbC, NMRI). On the contrary, use of conditioned medium enabled us to derive ES cell lines from non-permissive strains as well. With regard to the number of embryos, efficiencies were the following: 129SV: 6%; F2: 2%; CBA: 6%; BalbC: 6%; NMRI: 17%.

Pluripotency of cell lines were studied by a series of in vitro tests. The degree of euploidy was determined counting the chromosomes following Giemsa staining. The gender of the cell lines was identified by PCR. Cell surface marker expression was studied using SSEA-1 immuno-staining. Activities of alkalic-phosphatase (AP) and Oct-4 transcription factor were detected. All cell lines were positive in all tests.

Following in vitro tests, cell lines were also evaluated in in vivo experiments. ES—diploid embryo aggregation chimaeras were constructed to see if the ES cell lines are capable of forming chimaeras and specially, germ-line chimaeras. All cell lines but the F2 could contribute to form germ-line chimaeras in aggregation chimaera experiments. The ratio of chimaerism, however, was different: R1 was similar to mstn$^{-/-}$/Egfp$^{+/+}$2, while mstn$^{-/-}$/Egfp$^{+/+}$1 and CBA were more capable to form chimaeras than R1. In case of mstn$^{-/-}$/Egfp$^{+/+}$1, the ability to form chimaeras was rather host-dependent: significantly more chimaeras were formed when CD1 was used compared to F2.

3.3 Genetic causes of strain-dependence

The genes having a role in the Oct-4 cascade were studied, namely: Oct-4, Sox2, Rex-1, Utf-1, Fgf4, and two others that play role in the maintenance of pluripotency: the receptor of LIF protein and the telomerase-reverse transcriptase. During the experiment, the cells were differentiated in medium lacking LIF for 10 days. The cells were cultivated in hanging drops for 2 days and in suspension for 8 days. Samples were analysed by semi quantitative RT-PCR. Rb7 served as inner control.

Thhe expression profile of Oct-4 during differentiation was very similar in all lines. It lacked in differentiated cells (fibroblasts) but it was present in non-

differentiated cells (e.g. ES cells). The amount of the transcript decreased during the first four days of differentiation and, by the 5th day, it could not be detected in the differentiating EBs. The Oct-4 was present in the embryos and in the ICM, as known from earlier studies. The expression level of Oct-4 was higher in the F2 and CBA cell lines than in R1.

The mRNA level of Sox2 slightly decreased during the differentiation of R1 and *mstn*$^{-/-}$*/Egfp*$^{+/+}$*2*. In other cell lines, no such decrease could be observed. In spite of the decrease of Sox2 expression, the total amount of the transcript was significantly higher than that of the Oct-4 and was still detectable on the 10th day. The Sox2 transcripts could be detected in both controls. In spite of the decrease of the expression of the Sox2, the overall amount of the transcript was significantly higher than that of the Oct-4 and it could be detected on the 10th day. Sox-2 transcripts could be detected in both controls.

The Fgf4 activated by the Sox2 and Oct-4 complexes showed a decreasing expression profile during differentiation. On the 8-10th day, it was not found in the cell lines of R1 and *mstn*$^{-/-}$*/Egfp*$^{+/+}$*2*, but was hardly detectable in F2 and CBA EBs.

The expressions of Rex1 and Utf1 showed very similar patterns in all differentiating cell lines. The level of expression was slightly declining. In the course of differentiation, no difference could be found between the five cell lines. The gp 190 subunit of the Lif receptor, that all RT-PCR primers were designed for, was present in all cell lines. This expression decreased in the last third of differentiation (7-10th day) in R1 and F2 but not in other cell lines.

The mRNA expression of the telomerase reverse transcriptase was also investigated. With the exception of the F2 line, it was detectable in all cell lines. No difference could be detected between cell lines. No mRNA expression of the telomerase was observed in the fibroblast.

3.4 Differentiation of ES cell line into skeletal muscle

Following the verification of the pluripotency of the newly established myostatin mutant ES cell lines, the differentiation ability of the cell lines was studied. All the three myostatin mutant cell, the control and the R1 cell lines have been differentiated. To differentiate in groups, the EBs were placed on gelatine-coated 10 cm Petri dish. The differentiation into skeletal muscle was characterised by the number of contracting clump of cells, the time and length of contraction and the groups of differentiated muscle cells.

No significant difference was found in the differentiating ability between cell lines, when the number of differentiated cells on the 30th day was counted. However, difference was observed in the process of differentiation. The time of appearance of the first contracting cell clumps and their number differed. Cells of mutant lines began to contract later (by the 10th day, their number increased) but longer: by the 15th day, their number was significantly higher. This was not observed in control cell lines.

The expression of MyoD, one of the earliest endogenous MRFs, was observed earlier in differentiating *mstn*$^{-/-}$*/Egfp*$^{+/+}$*2* mutant ES cells than in control

ones (*mstn*$^{+/+}$*/C7*). The expression profile was also extended, while the amount of mRNA was not shown to be higher. We note that the expression level of MyoD was slightly higher in *mstn*$^{+/+}$*/C7* control ES cell line compared to R1 line. The difference does not seem to be high but it was consistent through repeated experiments.

The expression of Myf5 appeared at the same stage with the same intensity as MyoD (D5), and no differences were observed between the cell lines. We observed a moderate difference in intensity between the 2 control ES cell lines on Day 16, 19, and 22: the level of the Myf5 transcript was higher in *mstn*$^{+/+}$*/C7* control line than in R1 cell line.

In case of myogenin and MRF4, a higher amount of transcript was observed during the differentiation of the *mstn* mutant cell line. The expression could be observed first on Day 13 in both cases but at a higher level than in the control lines. The expression of MRF4 was significantly prolonged compared to the *mstn*$^{+/+}$*C7*.

The expression level of Cdk2 in *mstn* mutant ES cell line was significantly higher, and the high expression period was longer than in the controls. The maximum duration of high expression was longer than eight days (D10, D13, and D16) in *mstn* mutant, meanwhile, high expression was identified only at one time point in controls (D10). The p21 expression was significantly lower but more persistent in the mutant *mstn*$^{-/-}$*/Egfp*$^{+/+}$*2* cell line.

The level of Smad3 transcript was slightly different in the myostatin mutant *mstn*$^{-/-}$*/Egfp*$^{+/+}$*2* ES cell line compared to the control ones: the transcript was not detectable on Day 16; otherwise, the level of transcript and the duration of the expression were equal. It should be remarked, that the detection of the Smad3 transcript was difficult on Day 5 in the mutant cell line.

Both Pax3 and Pax7 were different from the control in intensity of expression. Both genes were up-regulated in *mstn* ES cell lines during skeletal muscle differentiation. In case of Pax3, Day 5 and Day 10 expression levels decreased in *mstn*$^{+/+}$*/C7* cell line compared to R1.

The expression of Igf1 was also up-regulated in latter stages (D19). In case of MNF isoforms, no differences could be identified in the expression profile (nor in duration, neither in intensity) comparing the differentiation of myostatin mutant and control line.

The activity of Sox5 was different in the cell lines compared. The expression level of Sox5 transcript was higher at three time points in *mstn*$^{-/-}$*/Egfp*$^{+/+}$*2* cell line during ES cell differentiation (Day 10, 13, and 16). We also detected a difference between the two control cell lines: the expression of Sox5 was more delicate in R1 than in *mstn*$^{+/+}$*/C7*.

To determine the amount of structure genes transcripts, two different structural protein-coding genes were selected: desmin and myosin heavy chain (MyHC) isotypes. Desmin accumulates early in myoblasts and muscle cells during differentiation. The level of desmin transcripts was also up-regulated in the mutant cell line compared to the control in latter stages.

Muscle fibre type differentiation was also screened in the experiment. We used the slow type MyHC (MyHC I) and one of the fast types (MyHC IIb) to identify and analyse the fibre-specific expression. While the expression of MyHC I increased dramatically in differentiating *mstn* mutant cells, the expression of MyHC type IIb was lower compared to control. The decrease of MyHC IIb transcript level was moderate compared to the increase of the MyHC I transcript level. The high expression of MyHC I was permanent from Day 19 to the last time point examined.

3.4.1 In vitro differentiation os muscle derived stem cells (satellite cells) and primary myoblast cultures

Muscle-derived satellite cells were isolated from mutant (*mstn*$^{-/-}$*/Egfp*$^{+/+}$) and control (*mstn*$^{+/+}$*/C7* and *129SV* background) animals and were differentiated in vitro. The stem cell nature of cells was demonstrated by in vitro differentiation to fused, multinuclear, contractile stage and by monitoring their expression profile.

Expression patterns of the mutant and control cell cultures were compared (Figure 4). The level of MyoD, Myf5, Cdk2 and Sox5 were up-regulated in differentiating myoblasts (Day 3), while p21 and Smad3 were down-regulated. In the fusion stage (Day 7), the level of myogenin, MRF4, Cdk2, Igf-1, MyHC I, and Sox5 were up-regulated. Parallel with this, the down-regulation of Smad3 and Pax7 was observed in differentiating *mstn* mutant muscle satellite cell cultures as compared to control ones. In the last stage (Day 10), when fused, multinuclear and mostly contractile cells were analysed, the differences between the cell cultures decreased: only myogenin, MRF4, desmin and MyHC I were up-regulated in *mstn* mutant satellite cells.

Primary muscle cultures were prepared from 10-week-old animals (*mstn*$^{-/-}$ */Egfp*$^{+/+}$, *mstn*$^{+/+}$*/C* and *129SV*). The primary cultures confirmed the differentiation of muscle-derived stem cells because of their very similar expression patterns.

3.5 Analysis of bodyweight and muscle weight

Detailed morphological and histological analyses were performed in the *compact* strain. Litter and average individual weights, of myostatin mutant and control mice were compared at birth, at weaning and in adults. Weights of several muscles were also measured and compared.

Body- and muscle weight of the myostatin mutant mouse strain were measured. No significant difference was found in litter weight at birth, in corrected litter weight, in average bodyweight (calculated from litter weight) between myostatin mutant and control population. However, the difference between the control and the myostatin mutant animals was significant in corrected weaning weight and in bodyweight measured at the age of 16 weeks. A significant sex-dependent difference was found in the bodyweight at 16: the *mstn*$^{-/-}$ males weighed in average 51.57±2.18 g, being significant heavier than the controls (43.41±3.41 g). The situation was similar in females: the weight of the control animals (29.24±4.11 g) was inferior to that of the mutants (38.18±2.31 g). If gender was not

taken into account, there was no significant difference in bodyweight between the control (34.9±5.8 g) and the mutant (46.12±5.6 g) animals at the age of 16 weeks.

Beside bodyweights, muscles themselves were also weighed. It is clear from the literature that in the different muscles of a healthy animal, the myostatin-level is different. Therefore, it could be presumed that in the absence of myostatin, the proportion of hypertrophy is not the same in different muscles. Significantly increased muscle weights were detected compared to the control. The most significant difference between the controls and the mutants was observed in *m. vastus lateralis* (threefold increase) and in *m. gastrocnemius* (twofold increase). In other muscles investigated, the dimension of change was 1.5-2-fold.

3.6 MyHC fibre type analysis

Based on the RT-PCR experiments, where an increase was identified in the amount of MyHC I mRNA during ES and muscle stem cell differentiation in mstn mutant cell lines, we studied the effect of myostatin on different types of muscle fibre analysing cross sections from four different muscles of mstn mutant and control animals. The muscles were selected according to their 'normal' myostatin expression level analysed previously. *M. pectoralis* was selected for its high myostatin expression, m. biceps femoris and m. gastrocnemius for its medium expression, while m. masseter for not expressing myostatin. To distinguish white and red fibre types, all samples were stained using the NADH-TR staining.

The contribution of fibre types to the total sectional number and variations among different muscles could also be observed. The proportion of type I fibres in total fibre number not changed (*m. pectoralis*), decreased (*m. biceps femoris*) or increased (*m. gastrocnemius, m. masseter*). In intermedier fibres, this parameter did not change in the masseter, decreased in the gastrocnemius and pectoralis and increased in the biceps f. muscles. Proportion of type IIb fibres decreased in the biceps and increased in the gastrocnemius and pectoralis muscles.

Mean cross sectional areas of individual fibres and their comparison to the controls clearly show that in mutant muscles (with the exception of type I masseter fibres), all fibre types gained volume (although, to different extent) via increase in their individual fibre cross-sectional areas. The highest increase was observed in type I slow fibres in most cases.

Contribution of fibre types to the total sectional fibre area again shows variations. It is obvious in both controls and mutants that the largest subcompartment of the total fibres is type IIb, followed by intermedier fibres and type I fibre. Again, the type I fibres showed an increase in the contribution of total sectional area in all muscles examined.

3.7 Histological analysis

The structure of mstn mutant animals were investigated by light microscopy histotechniques and compared with those of a control animal. Samples were fixed

with formalin and embedded in paraffin. The sections were stained as mentioned earlier. Our findings were as follow:

- Following a staining with hematoxilin-eosin, an unequivocal increase could be detected in fibre diameter in mstn mutant animals. The increase of the fibre diameter apparently affects every fibre, however, specific fibre staining can only prove its extent.
- The cross sectional structure (fibre structure) did not differ when hematoxilin-eosin staining was used.
- The number of nuclei seemed to be higher in the photographs taken from cross-sections, maybe due to the plane of the cut, although it cannot be stated without specific investigation.
- Central nuclei and split fibres typical of the hyperplasia could be identified in muscles of *mstn*$^{-/-}$ mice.
- Vertical section stained with iron-hematoxilin showed normal striated muscle.
- Trichrome staining (azan, Van Gieson) revealed normal fibre structure.
- Trichrome staining and silver impregnation showed that there are les connective tissues in the muscles of *mstn*$^{-/-}$ animals.
- PAS staining revealed an increased amount of glycogen in the cross sections of mutant muscles.

In investigations using TEM, the ultrastructures of the muscles were studied on vertical and cross sections. Comparison of muscles of hypertrophic animals with those of the control ones revealed the following differences in ultrastructure:

- No pathological alteration was detected in myofibrillum structure in any of he muscles investigated.
- The number of mitochondria increased significantly in myostatin mutant muscles, especially in the region of the subsarcolemma. Here, the mitochondria piled up right under the membrane.
- Mitochondria of mutant and control animals differed not only in number but in size as well: several giant mitochondria were observed in myostatin mutant muscles.
- No pathological alteration was visible in the structure of mitochondria.
- Nuclei and satellite cells were seen more often in sections of mutant muscles than in controls. However, this phenomenon should be verified in further experiment.
- Tubular aggregates (TA) were visible in cross sections of mutant muscles.

3.8 Investigations on muscle cultures of myostatin mutant Belgian White Blue

Muscle precursor cell cultures have been isolated fom Belgian White Blue with pen muscle biopsy from *m. glutaeus intermedius*. *Holstein-Friesian* served as controls. The isolated cell cultures have been characterised and differentiated in vitro. The expression pattern of 13 genes related to the myostatin cascade has been

analysed. The genes were chosen based on the results of changes of gene expression. Our goal was to use and validate the information obtained from a mouse, a genetic model animal in cattle.

Satellite cell and primary myoblast culture have been produced successfully from biopsied muscle tissue. A significant increase was observed in the expression of MRF factors, Igf-1, Cdk2, while the level of p21 decreased significantly in *Belgian White Blue* cell cultures. In case of muscle fibres differentiating in vitro, the investigation of MyHC isotype revealed an MyHC IIB mRNA excess for the typical of adult animals in vivo for the expense of the IIA type. The differences measured in gene expression during differentiation underline the role of myostatin in the differentiation of the muscle tissue.

3.9 The new scientific results of the experiments are the following:

1. Es cell lines have been derived from myostatin mutant mouse strain. Their pluripotency has been verified *in vitro* and *in vivo*.
2. A new medium has been developed and the method has been modified for derivation of ES cell lines. This enabled the derivation of ES cell lines from non-permissive mouse strains. The pluripotency of these cell lines has been proven *in vitro* and *in vivo*.
3. It was found that in myostatin mutant mouse-derived ES cells differentiating in muscle, the myostatin decreases the expression of genes of MFR factors, Cdk2, Pax3, Pax7, Igf1, desmin, and Sox5 and increases the expression of p21 and Smad3.
4. The data of gene expression have been verified by parallel differentiation of mouse and bovine muscle stem cells and primary myoblast cultures.
5. Detailed histopathological analysis has been made in the comparison of myostatin mutant and control animals. Investigations by light microscopy and TEM revealed mitochondriopathy and forming of early tubular aggregates. The increased diameters of muscle fibres in hypertrophic muscles have been analysed and it was found that the number and the cross section area of the white type fibres increased. This was reinforced by *in vitro* gene expression analysis.

4. Discussion

Through the isolation of ES cell lines from an *mstn* mutant mouse strain, we aimed to obtain more information about the effect of myostatin on muscle differentiation. To reveal differences in cell differentiation and to follow *in vivo* cell movements, myostatin mutant mice were bred to become also *Egfp* transgenic and $mstn^{-/-}Egfp^{+/+}$. ES cell lines were isolated from these animals.

Although several ES derivation methods have been elaborated in the past decade, no efficient, easily executable and strain-independent method exists. Therefore, we aimed to develop a method that enables tu derive ES cell lines from non-permissive or non-inbred strains.

A new medium was developed in our laboratory. The new medium, collected from cultivation of ES cells, is used as a supplement to the traditional medium. The method is successfully used in deriving ES cell lines from myostatin-mutant inbred mouse strain (*Compact*). Not only a new medium but also a modified isolation method has been developed. This new and easily reproducible method, using the conditioned medium, can be used successfully in any strain. This method is an effective tool in tracking polygenic phenotypes in mutant strain.

Beside myostatin mutant mouse strain, permissive and non-permissive mouse strains were also used for testing. ES cell lines have been successfully derived from CBA, BalbC, and NMRI strains (non-permissive strains). Following derivation, their pluripotency was proven in details that enables to use them in cell biology and embryology.

Our newly established ES cell lines and the well-characterised control R1 line of 129SV origin were differentiated in vitro and expression of several genes responsible for early differentiation and cell lineage were observed. It was supposed that during the in vitro differentiation of cell lines of different genetic origin, the analysis of the activity of certain genes active during early commitment may reveal differences between strains and also the cause of strain-dependency.

The Oct-4 early transcription factor responsible for pluripotency and the genes playing a role in its signal pathway have been studied. Other important genes (such as telomerase or Lif receptor) that may have a role in stem cell renewal or in persistence of pluripotency were also involved in the study.

The decrease of the Oct-4 expression level was observed in differentiating EBs. Differences were found in the amount of Oct-4 between cell lines: in F2 and CBA lines, the level of expression was higher. It is assumed to be the cause of the fact that the efficiency of embryonic chimaera formation in these lines is low.

It6 is well known that the mRNA-level of Oct-4 influences the activation path of Oct-4. If it is high, the ES cells differentiate into endoderm and mesoderm; while low mRNA-level favours the differentiation into trophectoderm. Only the ‘normal’ level of mRNA can preserve the pluripotency of ES cells. Therefore, the differences in Oct-4 expression level between cell lines may explain the different pluripotency and in vivo chimaera-forming ability of embryonic cell lines.

In Fgf4, whose expression is activated by Sox2-Oct4 complexes, a slow decrease could be observed in the mRNA-level. Minor differences could be detected between cell lines: in the later stage of the differentiation, the R1 and *mstn*$^{-/-}$/*Egfp*$^{+/+}$*2* EBs lacked mRNA but F2, CBA and *mstn*$^{-/-}$/*Egfp*$^{+/+}$*1* did not. This variance could be a sign of strain-differences.

No difference was found in expression patterns of Utf1 and Rex-1 between the six ES cell lines. The expression of LIF receptor, member of the IL-6 cytokine family, was also investigated in differentiating ES cells. The signal pathway of the LIF, one of the main proteins in maintaining the pluripotency of ES cells, leads through the LIF receptor complex. Thus, the presence of the receptor signs if the ES cell was receptive or not, although, the LIF receptor subunit (also known as gp 190) is also a part of other IL-6 cytokine receptors. It means that the presence of the receptor is predictable but the activity of the LIF receptor can not be proved.

According to our observations, all our ES cell lines are LIF- and fibroblast-dependent: they can not be maintained in vitro without an embryonic fibroblast feeder layer. The mRNA of the LIF receptor is needed to maintain normal proliferation and pluripotency. The amount of transcripts of the LIF receptor showed a very similar pattern in all cell lines, its level not changed much during the 10 days of differentiation. It changed only in R1 and F2 lines between Day 7 and Day 10 compared to Day 0.

The necessity of telomerase reverse transcriptase in mouse ES cells is still a matter of debate. In our experiments, some telomerase activity was observed in all cell lines but F2 (where no telomerase reverse transcriptase could be detected). Activity was also detected in differentiating and proliferating EBs as well. No difference was found in telomerase activity of the four cell lines. The lack of telomerase mRNA can be responsible for the non-chimaera-forming nature of the F2 cell line. Since there is no unequivocal evidence for a direct role of the telomerase in the pluripotency of mouse ES cells, further experiments are required to provide evidence for this hypothesis.

It seems that the different expression patterns observed in ES cell lines can be responsible for the differences found between mouse strains. However, ES cell lines derived from other strains and comparative studies of the embryos are needed to give an unambiguous answer. In any case, our results confirm that the parameters of the pluripotency test can not be decreased and in vivo chimaera analysis is indispensable.

To study muscle differentiation, two stem cell differentiating systems were used. The *in vitro* ES cell differentiation procedure using embryoid bodies proved to be very effective in several experiments. The other was the i*n vitro* differentiation of muscle-specific stem cell population (i.e. satellite cell) was also widely used to obtain more information about the role and differentiation capabilities of stem cells. To our knowledge, this was the first study, where the differentiation capacity and expression pattern of differentiating ES cells to a special lineage (skeletal muscle) and somatic stem cells (satellite cells) were

analysed in parallel. Our in vitro data gained from these experiments underline the *in vivo* gene expression patterns.

The differentiation capabilities of the cell lines were also examined, and no differences were observed. However, the timing of the muscle-specific ES cell line differentiation was slightly different: in myostatin mutant cell lines, the appearance of contracting cells was delayed as compared to the R1 control and the 'contracting periods' of cells were maintained longer in the mutant lines. These differences were significant compared to the control. Our findings indicate that myostatin has an influence on the timing of muscle differentiation, both on cardiac precursor cells and skeletal muscle differentiatio.

In order to analyse the effect of myostatin, gene expression profiles were compared. A wide range of genes was selected to compare their expression pattern in natural *mstn* mutant and control stem cells. Altogether, 17 genes were selected for analysis. The genes involved in our studies were selected based on previous myostatin-related studies. A sequence analysis was also performed to identify transcription factor binding sites on myostatin flanking sequences.

The in vitro differentiation of ES cells into skeletal muscle was investigated with semi-quantitative RT-PCR. The differentiation was compared to muscle stem cell (satellite cell) differentiation as well. Gene expression pattern differences were found in MRFs expressions of the *mstn* mutant and control lines in both ES and satellite cells. The expression profile of the control cells was comparable to their embryonic expression pattern observed in the embryo during muscle cell differentiation.

The earlier and extended appearance of MyoD expression in mutant cells indicates that MyoD expression is not influenced by myostatin in early stages (between D5 and D10). It might also indicate that MyoD can trigger the expression of myostatin. In case of Myogenin and MRF4, a down-regulating effect was also observed: in the mutant, the lack of myostatin leads to an increase in the level of both transcripts. The high number of myogenin binding sites characterised on myostatin upstream sequences might allow myogenin to act on myostatin regulation as well.

In conclusion, we suppose that MRF and myostatin expression are regulated conversely. The predicted MyoD, Myogenin and Smad3 binding sites at the 5' and/or 3' regulatory region of myostatin might allow the corresponding factors to trigger the activity of myostatin. Nevertheless, DNA-protein binding assays should be performed to prove this relationship.

The increased transcript level of MRFs in *mstn* mutant cell lines suggests the down-regulation of these genes by myostatin in normal cells. However, the early MRFs (MyoD and Myf5) are expressed earlier in differentiating ES cells than myostatin mRNA could be detected. These two opposite actions can occur in parallel only if the MRFs regulate the myostatin gene activation but the level of myostatin serves as a feedback mechanism. From the available data, we could not conclude whether the level of MRFs or the level of myostatin acts as a feedback.

However, MyoD or myogenin knockout mice might offer a model to further analyse the expression and regulation of myostatin.

Two genes involved in cell cycle machinery were also examined in *mstn*$^{-/-}$ stem cells. Cdk2 was up-regulated, while p21 was down-regulated in both mutant stem cells. The results prove that myostatin has an effective role in directing proliferation: when myostatin bears mutation (e.g. in *mstn*$^{-/-}$ ES or satellite cells), the arresting effect does prevail, therefore, the decreased level of p21 is not capable to inhibit Cdk2. Furthermore, the increased level of Cdk2 can trigger cell proliferation by the phosphorylation of Rb protein. The paired homeobox transcription factors are both essential for satellite cell development and have a main role in driving muscle differentiation (In the *mstn* mutant, both Pax3 and Pax7 were up-regulated, which underline that myostatin can act as a negative regulator of proliferation.

The most remarkable difference was detected in Sox5 expression level. The fact that a gender-determining factor has binding sites at the myostatin locus may explain the previously observed gender-specific differences in the myostatin phenotype. While a significant increase of the Sox 5 mRNA level was detected in early stages of differentiation in mutant cells, it was not detectable in control cells. This expression pattern may explain the diverse phenotype of male and female myostatin mutant animals. There is not enough information for the clarification of the role of the Sox5, as the mice carrying the null-mutant variant of the gene die during embryogenesis and its role in testis development and in sexual determination can not be studied. Therefore, the increase in the level of Sox5 expression observed in myostatin mutant cell cultures can be presumed to play a role in the sexual determination of the phenotype because of the testis- and spermatid-specific expression of Sox5. However, the causes of appearance of gender-influenced phenotype require further research.

The expression profile observed in differentiating satellite cells was consistent with our ES cell line differentiation data. No difference was observed in intensity and length of expression between genes studied. Satellite cell differentiation studies confirmed the skeletal muscle-specific differentiation of the ES cells.

In our experiments, the three basic fibre types were identified by histochemical reaction of NADH-tetrazolium reductase (NADH-TR). Since no quantitative results of fibre composition of major muscles are available in normal or mutant mouse, and fibre diameter in histological studies show significant variances, the changes in fibre diameter due to lack of myostatin were proven by morphometry. The lack of myostatin also results in an increased —although to different degrees— diameter of muscles in mutant mice. Likewise, a significant increase in the amount of type I fibres could be observed in all muscles when proportions of different fibre types were investigated. This also underlines the elevated MyHC I expression detected in differentiating ES cells.

The results of the histopathological analyses agree in several cases with the alterations observed in hypertrophy or hyperplasia. The increased fibre diameter,

split fibres, the presence of central nuclei observed in hypertrophy also belong here. Decreased amount of connective tissues observed in myostatin null-mutant mice beside light microscopy histological analysis was observed in Cmpt strain as well. No other pathological alteration was detected in the ultrastructure of the fibres. PAS staining refers to elevated amount of glycogen. This can be in connection with fibre type that will be evaluated later.

TEM observations confirmed the intactness of the structure of myofibrillum. The increased amount of mitochondria observed together with myostatin mutation was unknown in the literature. This phenomenon usually appears in mitochondriopathy that often comes with structural alterationsis. In our case, however, no degradation of the crista was detected and the structures of the mitochondria were intact. Other alterations included formation of tubular aggregates (TA) ion the muscles of the mutant animals. This phenomenon was known in myopathy in human and mouse. Although, the number of animals investigated by TEM was low, it seems that these phenomenon are related with myostatin mutation and the double muscle phenotype.

Our results were compared with in vitro differentiation of hypertrophic bovine satellite cells and primary myoblasts in order to evaluate the results obtained from the mouse model. Although, it seemed that model animal serves well its purpose, there are differences between the model and the modelled animal due to phylogenetic and physiological differences.

Based on previous muscle differentiation experiments and literature data, there was no sign of difference in the genetic program of muscle development in cattle and mouse. It was presumed that from mouse experiments, valuable conclusions could be drawn.

To demonstrate this, somatic stem cells and primary muscle cell cultures were studied in Belgian White Blue cattle. The reason for using that breed was that (1) the hypertrophy is caused by a mutation of the myostatin, and (2) this breed can be found in Hungary in pure-blood. With the help of the national breeding association, mature sires were chosen from the animals of the Petőfi Mgtsz in Ostffyasszonyfa. Satellite cell and myoblast cultures were derived simultaneously from samples biopsied from gluteal muscle. Following the digestion of the tissue using a mild enzymatic treatment, the very small satellite cells were separated at the first filtering. After further treatments, myoblasts were isolated from the debris. Thus, cells could be obtained from biopsy, very efficiently, in one step.

Satellite cells and myoblasts were differentiated simultaneously. Similar to mouse experiments, mRNA samples were collected during in vitro differentiation and expression of several genes was studied. Results showed close correlation with those obtained from experiments with muscle differentiation of ES cell lines, somatic stem cells and primary myoblast cultures derived from myostatin mutant mouse. It can be concluded that there is no difference in the factors studied in the muscle differentiation cascade regulated by myostatin between the two species. Therefore, our experiments performed on the myostatin mutant mouse model well the changes observed in the Belgian White Blue breed.

5. Publications of the author related to the main topic

Periodical in foreign language, impact factor: >1

1. **Kobolák, J.,** Réz, G. and Gócza, E. (2004): Differences in gene expression in myostatin mutant ES cell lines during muscle differentiation *Cell Tissue Res*. (revised) **IF:2.991**

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2. **Kobolák, J.,** Gócza, E. (2002): The role of the myostatin protein in meat quality - a review. Arch. Tierz., 45(2):159-170. **IF.: 0,251**

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