Genomics 92 (2008) 474–477

Contents lists available at ScienceDirect

Genomics

journal homepage: www.elsevier.com/locate/ygeno

Identification of a missense mutation in the bovine *ATP2A1* gene in congenital pseudomyotonia of Chianina cattle: An animal model of human Brody disease

Cord Drögemüller [a,*], Michaela Drögemüller [a], Tosso Leeb [a], Francesco Mascarello [b], Stefania Testoni [c], Marco Rossi [d], Arcangelo Gentile [d], Ernesto Damiani [e], Roberta Sacchetto [b]

[a] *Institute of Genetics, Vetsuisse Faculty, University of Berne, Bremgartenstrasse 109a, CH-3001 Berne, Switzerland*
[b] *Department of Experimental Veterinary Sciences, University of Padua, Italy*
[c] *Department of Veterinary Clinical Sciences, University of Padua, Italy*
[d] *Veterinary Clinical Department, University of Bologna, Italy*
[e] *Department of Experimental Biomedical Sciences, University of Padua, Italy*

ARTICLE INFO

Article history:
Received 6 July 2008
Accepted 31 July 2008
Available online 25 September 2008

Keywords:
Cattle
Chianina
ATP2A1
SERCA1
Brody disease
Animal model

ABSTRACT

Congenital pseudomyotonia in Chianina cattle is a muscle function disorder very similar to that of Brody disease in humans. Mutations in the human *ATP2A1* gene, encoding SERCA1, cause Brody myopathy. The analysis of the collected Chianina pedigree data suggested monogenic autosomal recessive inheritance and revealed that all 17 affected individuals traced back to a single founder. A deficiency of SERCA1 function in skeletal muscle of pseudomyotonia affected Chianina cattle was observed as SERCA1 activity in affected animals was decreased by about 70%. Linkage analysis showed that the mutation was located in the *ATP2A1* gene region on BTA25 and subsequent mutation analysis of the *ATP2A1* exons revealed a perfectly associated missense mutation in exon 6 (c.491G>A) leading to a p.Arg164His substitution. Arg164 represents a functionally important and strongly conserved residue of SERCA1. This study provides a suitable large animal model for human Brody disease.

Exercise-induced muscle stiffness is an uncommon and non-specific disorder. Exercise-induced contractures occur in muscle glycogenosis and may belong to the spectrum of myotonia [1]. Recently, a new congenital muscular disease has been reported in Chianina cattle designated as pseudomyotonia (PMT) as opposed to true myotonia; in fact, the electromyographic examination does not show any alteration, not even during the phase of the muscle contraction [2]. The bovine disease is clinically characterized by a lifelong history of exercise-induced muscle contracture, which prevents animals from performing muscular activities more intense than a simple walk at a slow pace. The disease does not affect life expectancy. When the animals are stimulated to move fast, the muscles immediately become stiff giving a picture of permanent contraction; the stiffness disappears as soon as the exercise ceases (Fig. 1). Muscle biopsies taken from PMT affected cattle after muscle exercise showed necrotic and regenerative fibers in type 2 muscle fibers [2]. The observed PMT phenotype in Chianina cattle closely resembled the symptoms of human Brody disease [3], a rare genetic disorder (OMIM 601003) of muscle function. Brody disease is characterized by painless muscle contracture and exercise-induced impairment of muscle relaxation due to a defect of calcium reuptake. Mutations in the human *ATP2A1* gene, encoding a fast-twitch skeletal-muscle Ca^{2+}-ATPase (SERCA1), cause Brody disease [4–6]. However, similar to other muscle diseases, Brody disease in humans is genetically heterogeneous. In some families with recessively and dominantly inherited forms of Brody disease, no mutations in *ATP2A1* have been identified [7]. In most recessive cases, nonsense, missense, or frameshift mutations in *ATP2A1* result in deleted functional domains leading to loss of SERCA1 function of fast-twitch skeletal muscle fibers [4–6]. These findings explained earlier studies reporting reduced Ca^{2+}-stimulated ATPase activity and increased time required for return to basal calcium levels following depolarization-induced calcium release in skeletal muscle of patients with Brody disease [8].

Many inherited diseases of laboratory or domestic animals are analogous to human hereditary disorders and have proven to be valuable animal models both for the investigation of the pathogenesis and therapeutic trials of rare human phenotypes with identical molecular basis [9]. Recently, a missense mutation in bovine *ATP2A1* responsible for recessive inherited congenital muscular dystonia has been reported in Belgian Blue cattle [10]. The affected calves exhibit episodes of generalized muscular contractures and usually die within a few weeks as a result of respiratory complications. Unlike the human disease, in which patients survive and do not show signs of respiratory disease, *Atp2a1* null mutant mice exhibit a gasping respiration, become cyanotic, and die shortly after birth [11]. Taken together, until today no animal model with mutations in the *ATP2A1* gene is available, which reflects the mild course of human Brody disease.

* Corresponding author. Fax: +41 31 631 2640.
E-mail address: cord.droegemueller@itz.unibe.ch (C. Drögemüller).

0888-7543/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygeno.2008.07.014

Fig. 1. A 14 month-year old male PMT affected Chianina cattle during muscle exercise test showing bunny hopping in the hind legs.

Table 1
SERCA1 activity measurements

Sample	SERCA1 activity (μmol/min/mg protein)[a]
Control cattle[b]	
A	2.38±0.40
B	1.90±0.18
C	2.14±0.50
PMT affected cattle[c]	
No. 5	0.73±0.08
No. 8	1.05±0.07
No. 9	0.17±0.06

[a] SERCA1 activity of sarcoplasmic reticulum enriched microsomal fractions extracted from fast-twitch (type II) skeletal muscles was measured at 37 °C in the presence of calcium ionophore A23187. The given values are the mean (n=3)±S.D.
[b] Control animals were unrelated Chianina cattle.
[c] Numbering corresponds to Fig. 2.

In this study, we report the identification of a missense mutation in bovine *ATP2A1* in congential pseudomytonia of Chianina cattle, a suitable animal model for studying the human Brody disease.

Results and discussion

The pedigree of an established Chianina cattle family segregating for congenital pseudomyotonia (PMT) was consistent with monogenic autosomal recessive inheritance (Fig. 2). Analysis of the pedigree data from nine generations revealed that PMT occurred in different generations but all seventeen affected calves could be traced back to a single common male ancestor. In all cases, the parents were healthy and the latest recorded affected calf (animal No. 27; Fig. 2) was inbred from the probable founder sire as its parents had this bull as common ancestor 4 or 7 generations ago, respectively.

As the PMT phenotype in Chianina cattle shows striking clinical similarities to human Brody disease we hypothesized that mutations in *ATP2A1* might cause the bovine disease. In order to validate whether *ATP2A1* might be a reasonable functional candidate gene for PMT, we analyzed the function of the encoded SERCA1 pump in bovine skeletal muscles of affected and control cattle, respectively. In sarcoplasmic reticulum enriched microsomal fractions extracted from fast-twitch (type II) skeletal muscles, normal control cattle displayed a mean SERCA1 activity of approximately 2.14 μmol/min/mg protein and PMT affected cattle of approximately 0.65, respectively (Table 1). This functional assay indicated that fast-twitch skeletal muscles of PMT affected cattle showed only an activity of approximately one third of the normal values (Table 1). These data are in agreement with reduced sarcoplasmic reticulum SERCA1 activity in human Brody's patients, varying from 30% to 2% of controls [8,12]. In muscle biopsies from affected Chianina cattle no reduction in fibers diameters have been described [2]. Taken together, these results clearly demonstrated an obvious reduction of the molecular activity of SERCA1 in skeletal muscle of PMT affected Chianina cattle and confirmed its essential role in the pseudomyotonia phenotype.

Genotyping data of two microsatellite markers derived from the BTA25 sequence were used for linkage analysis in the presented family (Fig. 2). The markers were located approximately 170 kb upstream and 200 kb downstream of *ATP2A1*. The microsatellites had two and five alleles and the calculated polymorphism information content (PIC) was 19% and 25%, respectively (Suppl. Table 1). For linkage analysis, the pedigree was split into three sub-pedigrees because of inbreeding loops and the high proportion of missing DNA samples. The estimated nonparametric maximal Z mean value of 3.3 with $p<0.0005$ and the maximal parametric LOD score of 3.1 confirmed the linkage of PMT to the candidate gene region. All PMT affected calves showed homozygosity for both tested markers and all genotyped parents showed at least one copy of the disease associated haplotype. In two cases assumed carrier parents showed two copies of this haplotype and

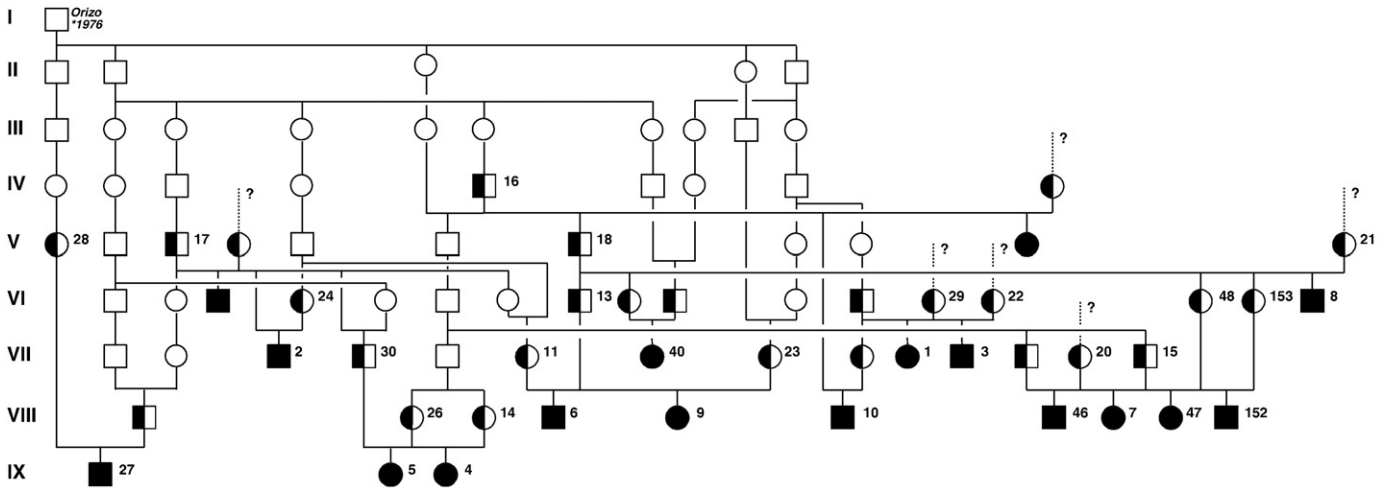

Fig. 2. Familial relationships between the 17 individuals of Chianina cattle affected with congenital pseudomoytonia (filled symbols). Half-filled symbols represent healthy obligate heterozygous carriers and open symbols represent healthy relatives with an unknown genotype. DNA samples were available from numbered animals. Dams marked with question mark indicate animals, where no breeding record was available.

their genotypes at both microsatellites were homozygous like in affected animals. Therefore, for breeding purpose an indirect PMT genotype prediction using these two flanking marker is probably problematic due to the low information content of these markers.

After we confirmed that the function of the encoded SERCA1 pump is compatible with a role in PMT in Chianina cattle and detected significant genetic linkage to the bovine *ATP2A1* gene, we initiated the mutation analysis. In order to search for possible causative mutations within *ATP2A1* the available bovine mRNA sequence (Acc. NM_001075767) was used for genomic sequence alignment with the current BTA25 sequence to characterize the exact gene structure. The bovine *ATP2A1* gene spans 16.5 kb and consists of 23 exons (Suppl. Table 2). All splice donor/splice acceptor sites conform to the GT/AG rule. The coding sequence of the bovine *ATP2A1* gene is contained within 22 exons encoding the transcript corresponding to human adult *ATP2A1* isoform b. The bovine SERCA1 protein is predicted to contain 993 amino acids with 95% identities to the human and murine SERCA1 proteins, respectively. Subsequently, we designed exon spanning PCR primer pairs (Suppl. Table 2). As the current bovine genome assembly (build 4.0) contained a gap including the entire sequence of *ATP2A1* exon 10, we submitted the obtained genomic sequence of a 526 bp amplified PCR product containing exon 10 and flanking intronic sequences into EMBL nucleotide database (Acc. FM177181).

Mutation analysis was performed in two trios of affected calves and their parents and in two unrelated controls of Chianina cattle. Affected cattle carried homozygous a single base transition within exon 6 (c.491G>A, Fig. 3a). The mutation affects an *Acc*I recognition site. The presence of the *ATP2A1* wildtype allele G will result in cleavage, whereas the mutant allele A will give rise to the absence of the restriction site. A second constitutive *Acc*I recognition site located in the amplified product serves as a positive control (Fig. 3b). No other mutations in exonic or splice site regions of the *ATP2A1* gene were identified in PMT affected animals. To check whether the observed missense mutation affects *ATP2A1* splicing we amplified the entire *ATP2A1* cDNA of three affected and a single control animal using four overlapping RT-PCR products (Suppl. Table 3). No alterations of the transcript could be observed apart from the c.491G>A polymorphism. We genotyped additional cattle for the exon 6 mutation and found perfect concordance between the presence of the mutation and the PMT phenotype in all 15 affected animals from the reported family, while homozygous animals for the mutant allele were not present in any of the investigated 18 healthy Chianina animals of the presented family nor in 58 other healthy unrelated Chianina breeding bulls. Among these 58 bulls, representing the current Italian Chianina population, 5 PMT carriers were observed, indicating a frequency of approximately 4% of the mutant *ATP2A1* allele. The PMT mutation has probably been introduced by the founder sire of the presented family, as until now all identified carriers can be traced back to this Italian Chianina artificial insemination bull named *Orizio* born in 1976. As artificial insemination caused a drastic reduction of the effective population size in Italian Chianina cattle, the recently reported outbreak of the recessive PMT diseases gives an example that the accompanying increase in co-ancestry and inbreeding could lead to a quite high incidence of a genetic disease within a short time. Therefore, we already informed the Italian Chianina breeders about the DNA-based test for the detection of PMT carriers and they already started to implement this information in their breeding strategy. This new gene test identifies the second breed specific mutation after the recent identification of the molecular cause of ichthyosis fetalis in Italian Chianina cattle [10]. Sequencing of 30 unrelated control animals, 10 bulls each of the cattle breeds Romagnola, Simmental, and Brown Swiss showed that the *ATP2A1* c.491G>A polymorphisms is not present in these three breeds.

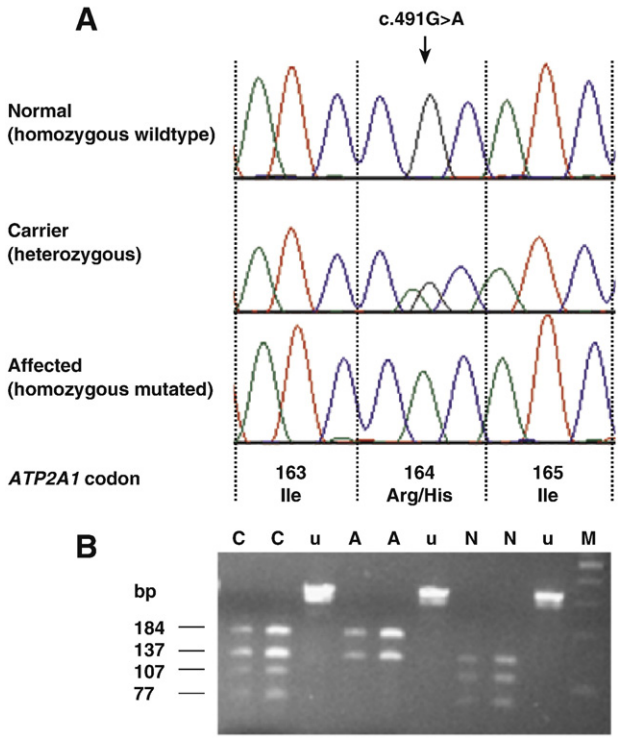

Fig. 3. Mutation analysis of the bovine *ATP2A1* gene. (A) Sequence analysis of genomic DNA of an affected, an obligate heterozygous carrier, and an unrelated control animal An arrow denotes the position of the mutation. Numbering of nucleotides and codons is according to the open reading frame of the *ATP2A1* cDNA sequence (Acc. NM_001075767). (B) RFLP analysis: After digestion of the PCR product with *Acc*I the wildtype G-allele results in 77, 107 and 137 bp fragments, whereas the mutant A-allele leads to 137 and 184 bp fragments. Two heterozygous carriers (C), two affected (A), and two normal control (N) animals are shown. The undigested (u) PCR product and a 100 bp ladder (M) are also shown.

The missense mutation leads to an amino acid exchange (p.A164H) within a highly conserved domain of SERCA1 and affects the N-terminal actuator domain (formerly called the transducer or beta domain), one of the three cytosolic domains, besides the phosphorylation and nucleotide-binding domains, which form the catalytic site involved in the transmission of major conformational changes during $Ca^{2+}$and ATP binding [13]. The potential impact of this PMT associated amino acid substitution was evaluated by multiple species alignments (Fig. 4) and PolyPhen (http://genetics.bwh.harvard.edu/pph/). The substitution affects a strongly conserved residue and was predicted to be “probably damaging” with a PSIC score difference of 2.94, which implies a high confidence that the substitution affects protein function or structure. It is still unclear whether the mutant protein has an activity and a residual physiological function. A reduction in SERCA1 activity in PMT affected skeletal muscle was observed and this is most likely due to diminished activity of the mutant SERCA1. By pumping the calcium ions from the sarcoplasma of the muscular fiber back into the sarcoplasmic reticulum, SERCA1 decreases the calcium concentration within the sarcoplasma, thereby enabling the relaxation of myofilaments. The deficiency of functional SERCA1 in the sarcoplasmic reticulum could explain the clinical presentation of the cattle. Progressive impairment of muscle relaxation during exercise would occur because of the cumulative increase in myofibrillar Ca^{2+}. Since only fast-twitch muscle fibers suffer from enzyme deficiency, it is plausible that impaired relaxation is only noted after phasic exercise (i.e. during strong exercise) when primarily fast-twitch motor units are utilized. Thus, the observed mutation most likely results in the inability of SERCA1 to keep pace with calcium release during repetitive stimulation. By contrast, during tonic activity (such as maintaining posture) or during slow paced walk requiring slow-twitch motor unit activation, no obvious muscular dysfunction is

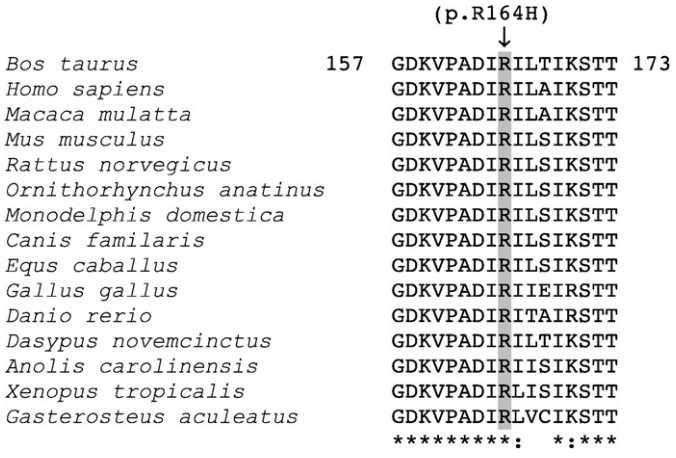

Fig. 4. Multiple sequence alignment of the SERCA1 protein in the region of the mutation demonstrates the conservation of arginine residue 164 (R164) across species. The observed mutation in PMT affected cattle is indicated by an arrow and the respective position is highlighted in grey. Identical residues are indicated by asterisks beneath the alignment, while colons and dots represent very similar and similar amino acids, respectively.

noted. Because muscle relaxation is slow but not completely impaired, compensatory mechanisms apparently clear myoplasmic calcium over time. These may include residual SERCA1 and compensatory SERCA2 activity of slow-twitch skeletal muscle, calcium extrusion via plasma membrane Ca^{2+}-ATPase pumps and Na^{+}/Ca^{2+}exchangers, as well as mitochondrial calcium uptake [14,15].

In summary, we have applied a candidate gene approach to identify *ATP2A1* as the causative gene for PMT in Chianina cattle and thereby discovered this new bovine genetic disorder as suitable large animal model for human Brody disease. The PMT affected cattle might be very helpful in the possible identification of new targets for therapeutic interventions, as e.g. through injections of recombinant SERCA1 protein. In addition, although the understanding of the pathogenesis of the disease is still incomplete, the unique bovine pseudomytonia phenotype provides additional information on the role of SERCA1. The developed *ATP2A1* gene test enables breeders of Chianina cattle to get rid of this genetic disease. Taken together, this study reflects the enormous potential of domestic animals to gain further insights into mammalian biology and human medicine.

Material and methods

A total of 17 Chianina cattle (9 male, 8 female) with the clinical diagnosis of congenital pseudomytonia were recorded. Most of these cases could be paternally and maternally traced back to a single common male ancestor of the established Chianina family, for the remaining animals pedigree information was incomplete (Fig. 2). Samples from 15 affected animals and, where possible, their sire and dam were collected and parentage verification was performed to ensure correct parentage. The parents of affected offspring were classified as obligate carriers. In total, 33 DNA samples of 15 affected animals and 18 parents were extracted from blood, tissue, or semen using standard molecular techniques. For RNA isolation, bovine skeletal muscle biopsies from an unrelated control and three affected animals were used for total RNA extraction and subsequent cDNA synthesis using standard molecular techniques.

Preparations of skeletal muscle sarcoplasmic reticulum of biopsies of *M. semimembranosus* from three PMT affected and three normal control animals and SERCA1 activity measurements in the membrane preparations assayed by a spectrophotometer method were performed as described before [16].

Microsatellite markers were amplified using the Multiplex PCR Kit (Qiagen) and fragment size analyses were determined on an ABI 3730 capillary sequencer (Applied Biosystems, Rotkreuz, Switzerland) and analyzed with the GeneMapper 4.0 software (Applied Biosystems). Multipoint nonparametric linkage and parametric linkage analyses were performed with MERLIN software version 1.1.2 [17]. For parametric linkage, we calculated LOD scores under both homogeneity and heterogeneity under the assumption of PMT segregating as a biallelic autosomal recessive trait, with complete penetrance. The frequency of the defect allele in the considered population is unknown and there is no data available that would make it possible to estimate the frequency in a reliable manner. For the calculations a frequency of 0.01 for the mutated allele was assumed.

For the *ATP2A1* mutation analysis suitable PCR products were amplified using two affected, four heterozygous carrier, and two unrelated healthy animals using AmpliTaq Gold (Applied Biosystems) (Suppl. Table 2). The subsequent re-sequencing of the PCR products was performed after rAPid alkaline phosphatase (Roche, Basel, Switzerland) and exonuclease I (N.E.B., Bioconcept, Allschwil, Switzerland) treatment using both PCR primers with the ABI BigDye Terminator Sequencing Kit 3.1 (Applied Biosystems) on an ABI 3730. Sequence data were analyzed with Sequencher 4.6 (GeneCodes, Ann Arbor, MI, USA).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygeno.2008.07.014.

References

[1] S. DiMauro, C. Lamperti, Muscle glycogenosis, Muscle Nerve 24 (2001) 984–999.
[2] S. Testoni, P. Boni, A. Gentile, Congenital pseudomyotonia in Chianina cattle, Vet. Rec. 163 (2008) 252.
[3] I.A. Brody, Muscle contracture induced by exercise: a syndrome attributable to decreased relaxing factor, New Eng. J. Med. 281 (1969) 187–192.
[4] A. Odermatt, et al., Mutations in the gene-encoding SERCA1, the fast-twitch skeletal muscle sarcoplasmic reticulum Ca^{2+}ATPase, are associated with Brody disease, Nat. Genet. 14 (1996) 191–194.
[5] A. Odermatt, et al., Characterization of the gene encoding human sarcolipin (SLN), a proteolipid associated with SERCA1: absence of structural mutations in five patients with Brody disease, Genomics 45 (1997) 541–553.
[6] A. Odermatt, et al., The mutation of pro(789) to leu reduces the activity of the fast-twitch skeletal muscle sarco(endo)plasmic reticulum Ca^{2+}ATPase (SERCA1) and is associated with Brody disease, Hum. Genet. 106 (2000) 482–491.
[7] Y. Pan, et al., Targeted disruption of the ATP2A1 gene encoding the sarco(endo) plasmic reticulum Ca^{2+}ATPase isoform 1 (SERCA1) impairs diaphragm function and is lethal in neonatal mice, J. Biol. Chem. 278 (2003) 13367–13375.
[8] A.A.G.M. Benders, et al., Ca(2+) homeostasis in Brody's disease: a study in skeletal muscle and cultured muscle cells and the effects of dantrolene and verapamil, J. Clin. Invest. 94 (1994) 741–748.
[9] D.F. Patterson, et al., Research on genetic diseases: reciprocal benefits to animals and man, J. Am. Vet. Med. Assoc. 193 (1988) 1131–1144.
[10] C. Charlier, et al., Highly effective SNP-based association mapping and management of recessive defects in livestock, Nat. Genet. 40 (2008) 449–454.
[11] Y. Pan, et al., Targeted disruption of the ATP2A1 gene encoding the sarco(endo) plasmic reticulum Ca^{2+}ATPase isoform 1 (SERCA1) impairs diaphragm function and is lethal in neonatal mice, J. Biol. Chem. 278 (2003) 13367–13375.
[12] A.A. Benders, R.A. Wevers, J.H. Veerkamp, Ion transport in human skeletal muscle cells: disturbances in myotonic dystrophy and Brody's disease, Acta Physiol. Scand. 156 (1996) 355–367.
[13] D.H. MacLennan, N.M. Green, Structural biology. Pumping ions, Nature 405 (2000) 633–634.
[14] G. Karpati, J. Charuk, S. Carpenter, C. Jablecki, P. Holland, Myopathy caused by a deficiency of Ca^{2+}-adenosine triphosphatase in sarcoplasmic reticulum (Brody's disease), Ann. Neurol. 20 (1986) 38–49.
[15] A.E. Rossi, R.T. Dirksen, Sarcoplasmic reticulum: the dynamic calcium governor of muscle, Muscle Nerve 33 (2006) 715–731.
[16] R. Sacchetto, E. Damiani, A. Pallanca, A. Margreth, Coordinate expression of Ca^{2+}-ATPase slow-twitch isoform and of beta calmodulin-dependent protein kinase in phospholamban-deficient sarcoplasmic reticulum of rabbit masseter muscle, FEBS Lett. 481 (2000) 255–260.
[17] G.R. Abecasis, S.S. Cherny, W.O. Cookson, L.R. Cardon, Merlin-rapid analysis of dense genetic maps using sparse gene flow trees, Nat. Genet. 30 (2002) 97–101.