Restriction of Calpain3 Expression to the Skeletal Muscle Prevents Cardiac Toxicity and Corrects Pathology in a Murine Model of Limb-Girdle Muscular Dystrophy

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Abstract:

Background—Genetic defects in calpain3 (CAPN3) leads to Limb-Girdle Muscular Dystrophy type 2A (LGMD2A), a disease of the skeletal muscle that affects predominantly the proximal limb muscles. We previously demonstrated the potential of Adeno-Associated Virus (AAV)-mediated transfer of the CAPN3 gene to correct the pathological signs in a murine model for LGMD2A after intramuscular and loco-regional administrations.

Methods and Results—Here, we showed that intravenous injection of calpain3-expressing vector in mice can induce mortality in a dose-dependent manner. An anatomo-pathological investigation revealed large areas of fibrosis in the heart that we related to unregulated proteolytic activity of calpain3. To circumvent this toxicity, we developed new AAV vectors with skeletal muscle-restricted expression by using new muscle-specific promoters that include the CAPN3 promoter itself and by introducing a target sequence of the cardiac-specific microRNA-208a in the cassette. Our results show that CAPN3 transgene expression can be successfully suppressed in the cardiac tissue, preventing the cardiac toxicity, while expression of the transgene in skeletal muscle reverts the pathological signs of calpain3 deficiency.

Conclusions—The molecular strategies used in this study may be useful for any gene transfer strategy with potential toxicity in the heart.

Key words: gene therapy, death, sudden, muscle, microRNA
Introduction

Mutations in the calpain3 gene (CAPN3) cause one of the most common form of autosomal recessive limb girdle muscular dystrophy (LGMD2A; OMIM 253600). The proximal skeletal muscle impairment seen in this disease is associated with an elevation of serum creatine kinase and dystrophic features such as areas of degeneration/regeneration, variation in fiber size and inflammatory infiltrates. It is worth mentioning that cardiac and facial muscles are not affected.

Calpain3 is a member of the calpain family of non-lysosomal calcium-activated cysteine proteases. Although some isoforms were identified in lens and brain, the classical full-length form of CAPN3 is expressed specifically in the skeletal muscle. The enzyme is present in this tissue as a dormant enzyme and self-activates by autolysis through the removal of an internal peptide to free the catalytic site for substrate accessibility. The precise function(s) of calpain3 and the mechanism by which it causes LGMD2A are not fully understood although several evidences pinpoint a role in cytoskeleton remodeling.

In an attempt to develop a therapeutic strategy based on gene transfer for LGMD2A, we previously tested local injections of Adeno-Associated Virus (AAV) vectors expressing CAPN3 in skeletal muscle of calpain3 deficient mice. An efficient CAPN3 transgene expression in skeletal muscles with a correct localization at the sarcomere was observed. This expression was associated with a restoration of the calpain3 proteolytic activity and rescue of the contractile force deficits. In the present study, we tested systemic injection of calpain3 AAV vector and observed cardiac toxicity that we related to an unregulated proteolytic activity of calpain3 in the heart. Following these results, new AAV vectors were designed and validated in vivo for permitting CAPN3 transgene expression in skeletal muscles without showing any cardiac toxicity. Moreover, the expression level of the transgene in skeletal muscles was sufficient to
observe a therapeutic effect with correction of the myopathology. These results represent a step further towards the use of CAPN3 gene transfer as a therapeutic approach for LGMD2A patients.

**Methods**

**Plasmid construction and AAV production**

All vectors were constructed by classical molecular biology techniques (See Supplemental Methods). Plasmid DNA was prepared using the Maxiprep 500 EF kit (Macherey Nagel) and all constructs were verified by sequencing. Viral preparations were generated by packaging AAV2-ITR recombinant genomes in AAV1, AAV8 or AAV9 capsids by a triple transfection of HEK-293 cells and purified by Cesium chloride gradient centrifugation. Viral genomes were quantified by q-PCR as already described.

**Cell culture and transfection**

Human embryonic Retinoblasts (HER911), Human umbilical vein-endothelial cells (HUV-EC), Human fibrosarcoma cells (HT1080), Human cervix adenocarcinoma cells (HeLa) and Human hepatocellular carcinoma cells (HuH-7), Human embryonic kidney cells (HEK293) and C2C12 myogenic cell lines were obtained from the ATCC and cultured in DMEM complemented with 10% of Fetal Bovine Serum. They were transfected with 2 μg of plasmid for 6 μl of FuGENE-HD transfection reagent (Roche Applied Science, Indianapolis, IN, USA). C2C12 myoblasts were differentiated in myotubes for 5 days by changing medium composition (DMEM + Horse Serum 2%).

**Regular and quantitative PCR**

Muscle or cell lysates were processed using the Trizol method (Invitrogen). A fraction of the sample was kept for DNA extraction for qPCR quantification. Total RNA was extracted from the
remaining extract that was treated with Free DNA kit (Ambion). The details of assays and primers are presented in Supplemental Methods.

**Determination of murine Secreted Alkaline Phosphatase (mSeAP) activity**

After transfection of the various plasmids containing mSeAP in cells, 200 μl of the medium per well was removed for alkaline phosphatase (AP) activity measurement from days 0 to 5 of differentiation for C2C12 cells and 48 h after transfection for the human cell lines. Detection of AP activity was carried out with the Phospha-Light assay system (Applied Biosystems, Bedford, MA, USA). The data were normalized to the quantity of transfected plasmid measured by quantitative PCR (qPCR).

**Western blot**

HER911 were harvested 48h after transfection. Proteins were extracted using a lysis buffer [4M Urea, 20mM Tris-HCl (pH8.5), 5mM DTT, 1.5mM MgCl2, 1% TritonX-100, Benzonase (25U/ml, MerK), 2μM E64 (Sigma) and Complete mini protease inhibitor cocktail (Roche Applied Sciences)]. Muscles were homogenized using FastPrep and livers using Ultra Turrax T10 (IKA). The following lysis buffer [20mM Tris (pH7.5), 150mM NaCl, 2mM EGTA, 0.1% TritonX-100, 2μM E64 (Sigma) and Complete mini protease inhibitor cocktail; 25 μl per mg of tissue] was used. Samples were treated with 250U/100 μl of Benzonase (Calbiochem) for 30 min at 4°C to digest DNA. Lysates were mixed with loading buffer [NuPage LDS (Invitrogen), DTT 3M (Sigma)] and denatured for 10 minutes at 70°C. Supernatants were separated by 4-12% Bis-Tris gel polyacrylamide gradient NuPAGE gels (Invitrogen). After blotting, membranes were probed with antibodies against calpain3 (mouse monoclonal antibody, Novocastra NCL-CALP-12A2, dilution 1/200), GFP (ab-6556, rabbit polyclonal, Abcam, dilution 1/1,000), α-actin (A-4700, mouse monoclonal antibody, dilution 1/200 or A-2066, rabbit polyclonal antibody, Sigma,
dilution 1/400) or GAPDH (sc-25778, rabbit polyclonal antibody, dilution 1/800). Finally, membranes were incubated with IRDye® for revelation by the infrared-scanner Odyssey (LI-COR Biosciences, Lincoln, Nebraska, USA). Quantification of protein was performed with the software Odyssey 2.1.

**In vivo experiments**

C57BL/6 mice were purchased from Charles River Laboratories (Les Oncins, France). Construction and characterization of the C3-null mouse model (C3KO) was previously described\(^9\). The details and dose used for intravenous injections are presented in Supplemental Methods. The organs were removed and quickly frozen in liquid nitrogen-cooled isopentane or fixed with formol.

**Histology**

Cryosections were prepared from skeletal and cardiac muscles. Transverse sections were processed for Hematoxylin-Phloxine-Saffron (HPS) or Sirius red histological staining. Liver biopsies were fixed with formol and embedded with paraffin, prior to sectioning and processed for Hematoxylin-Eosin (HE). Morphometrical analysis to define the number of centronucleated muscle fibers (CNF/mm\(^2\)) were performed using Histolab software (Microvision, Evry).

For immunohistochemical detection of calpain3, unfixed transverse sections were blocked with PBS, FBS 3%, BSA 0.5% for 1 hour, then incubated with antibody against calpain3 (208757-Domain I, Merck-Millipore, dilution 1/200) and then with a goat anti-rabbit secondary antibody conjugated with Alexa 594 dye (Molecular probe, dilution 1/1000). Sections were visualized on a fluorescent microscope (Axioplan, Zeiss).

**Statistics analysis**

Data are presented as individual data + means. Individual measures were compared using the...
Mann-Whitney non-parametric test using Excel macros (http://herve.delboy.perso.sfr.fr/). Differences were considered to be statistically significant at \( P < 0.05 \).

**Ethical study approval**

All procedures on animals were performed in accordance with the directive of 24 November 1986 (86/609/EEC) of the Council of the European Communities and were approved by Genethon’s ethics committee under the number CE11-010.

**Results**

**Cardiac toxicity of CAPN3 transgene by systemic administration of AAV vectors**

The safety and efficacy of AAV-mediated CAPN3 gene transfer by intramuscular injection in a LGMD2A mouse model was previously demonstrated\(^7\). To go a step further toward therapeutic application for LGMD2A patients, we tested CAPN3 gene transfer by systemic administration. WT mice (\( n = 3 \) to 9) were injected in the tail vein with different doses \([4 \times 10^{12}, 1 \times 10^{13}, 1.6 \times 10^{13} \text{ and } 4.3 \times 10^{13} \text{ viral genome (vg/kg)}]\) of a AAV2/9 carrying the human CAPN3 cDNA under the control of desmin promoter (AAV2/9-desm-CAPN3 or pdes.C3). Prior to the scheduled end of the experiment, we observed mortality among the animals treated with the two highest doses since several animals died (**Supplemental Table A**). These sudden deaths led us to perform a detailed anatomo-pathological investigation of the animals. Large areas of fibrosis were detected in the heart of all the mice even those injected with the lower doses (**Supplemental Table A; Figures 1A and B**). Since AAV9 is known to transduce very efficiently the cardiac tissue, we tested two other AAV serotypes: AAV1 and AAV8. WT mice were injected with the maximal doses that we obtained for both viral preparations (AAV2/1: \( 1.6 \times 10^{13} \text{ vg/kg, } n=3 \) and AAV2/8: \( 7 \times 10^{12} \text{ vg/kg, } n=4 \)). Although death was observed only with AAV8 in two animals out of four,
destruction of the cardiac tissue was present in all animals whatever the serotype at the endpoint of the experiments 35 days after injection (Supplemental Table A).

To obtain direct evidence that this cardiac toxicity was related to ectopic calpain3 activity, WT mice were injected intravenously with an AAV vector expressing an inactive calpain3 protein mutated at the cysteine 129 (C129S) of the catalytic triad (AAV2/9-desm-CAPN3C129S or pdes.C3C129S, 1.6x10^{13} vg/kg, n=4). No sign of fibrosis was detected in the heart of mice injected with this vector compared to those injected with pdes.C3 (Figures 1A and B).

Transgene expression analysis by western blot showed high expression of inactive calpain3 in heart samples transduced with AAV2/9-desm.-CAPN3C129S, indicating an efficient gene transfer (Figure 1C). However, the active protein is undetectable in the heart samples injected with pdes.C3, in all probability due to the loss of the transduced fibers (Figure 1C).

**Generation of a miRNA-regulated cassette for down-regulation of CAPN3 transgene expression in cardiac tissue**

A strategy we used to solve the problem of calpain3 toxicity in the heart was to construct a microRNA-regulated vector. We identified miR-208a, which is encoded within intron 27 of α-cardiac muscle myosin heavy chain gene (αMHC), as a confirmed cardiac-specific miRNA. An expression analysis substantiated the specific expression of miR-208a in the heart and indicated that its expression is not modified by calpain3 deficiency or secondarily to the myopathology (Supplemental Figure 1A).

The target sequence of this miRNA was used to synthesize a DNA fragment carrying two copies of the sequence (miR208aT) in tandem. We then tested the capacity of the miR208aT to down-regulate the expression of calpain3 in cellulo (Figure 2). We performed a co-transfection experiment with a fluorescent plasmid expressing the precursor of miR-208a (premiR-208a) and
fluorescent plasmids expressing calpain3 in HER911 cells (see Supplemental Methods and Supplemental Figure 1B for the design of the constructs). Expression of the different transgenes was analyzed 48 hours after transfection by western blot and compared to the mRNA level (normalized by plasmid DNA level). The results showed that the exogenous miR-208a (DsRed-premiR208a) was able to strongly decrease the protein level of calpain3 only when miR208aT is present in the cassette (Figures 2A and B). Moreover, miR-208a seems to regulate the expression of calpain3 by blocking its translation rather than degrading its mRNA (Figure 2B). This result indicated that the tandem of miR-208a target sequence introduced in the cassette was efficient to suppress calpain3 expression in presence of miR-208a.

Cloning and characterization of new muscle-specific promoters

A second strategy was developed by changing the promoter to restrict the expression of the transgene to the skeletal muscles. In addition to reduce the cardiac toxicity of CAPN3 transfer it should also prevent the possibility of toxicity in other organs than the heart. We cloned the CAPN3 promoter itself and the human promoter of the skeletal muscle specific microRNA-206 (miR-206)\textsuperscript{11}, since both of these promoters are known to drive a skeletal muscle specific expression. As a first step, we performed an interspecies comparative analysis of the sequences upstream of the CAPN3 gene and miR-206 using the Evolutionary Conserved Region web platform to identify the conserved regions (ECR; http://ecrbrowser.dcode.org/; See Supplemental data and Supplemental Figures 2 and 3A for more details). Following this in silico analysis, we cloned, upstream of the mSeAP reporter gene, a 1650 bp fragment (hg19: chr15:42650353-42652003) for CAPN3 and a 818 bp region (hg19: ch6:52008326-52009144) for miR-206. An expression analysis performed in skeletal muscle (tibialis anterior; TA) and heart from WT and a calpain3 deficient animal model (C3KO mice) confirmed the specificity of expression of miR-
206 to skeletal muscles and that no modification of its expression occurred in C3KO mice due to calpain3 deficiency or in response to the myopathology (Supplemental Figure 3B).

The constructed reporter plasmids containing either CAPN3 or miR-206 selected DNA fragments were transfected in C2C12 cells to assess their transcriptional activity. The reporter gene expression was monitored by measuring mSeAP activity in the supernatant at myoblast stage and throughout differentiation in myotubes. We observed an increase of mSeAP activity from day 0 to day 5 of differentiation with the human miR-206 (miR206) and CAPN3 (C3) promoters. These results showed that the miR-206 and CAPN3 promoters are efficient in myotubes even if their promoting activities are lower than the desmin (Desmin) promoter (Figure 3A). The evaluation of endogenous CAPN3 and miR-206 transcripts levels during C2C12 differentiation was also monitored in parallel by RT-PCR. The cloned fragments promoting activity follows the expression curve of the endogenous miR-206 and capn3 mRNA (Figure 3B). An expression analysis on different human cell lines of the promoters ascertained their overall selectivity for muscle (Figure 3C and Supplemental data).

In vivo validation of regulation of the transgene expression in the heart

We then introduced the miR208aT in the AAV2/9 vector with the desmin-CAPN3 cassette (AAV2/9-desmin-CAPN3-miR208aT or pdes.C3-T) and constructed two CAPN3 AAV vectors by exchanging the desmin promoter by the CAPN3 (AAV2/9-pC3-CAPN3 or pC3.C3) or miR-206 (AAV2/9-pmiR206-CAPN3 or p206.C3) promoters. These AAV vectors were injected intravenously at a dose of 6x10^{12} vg/kg in two-month old mice (n=3 to 6). Mice were clinically monitored every day until sacrifice, 35 days after injection. Cardiac fibrosis was not observed in mice treated with the new vectors (pC3.C3, p206.C3 or pdes.C3-T) in contrast to the mice injected with pdes.C3 (Figure 4A), despite a similar or even higher level of transduction as
shown by the quantification of vector DNA copy number (Figure 4B). The CAPN3 transgene mRNA level in the heart for the mice treated with pdes.C3 was about 15-fold higher than the endogenous capn3 mRNA (Figure 4C). A five-fold higher mRNA level of CAPN3 transgene was observed in the heart of mice injected with pdes.C3-T compared to the endogenous level of capn3 mRNA, while it remained lower to the endogeneous level for the mice treated with pC3.C3 or p206.C3 (13% and 30%, respectively; Figure 4C).

Considering the high level of transcriptional expression with pdes.C3-T and in view of the translational control that was seen in cellulo, we undertook a protein analysis of the heart extracts (Figure 4D). In the WT mice, calpain3 is not expressed normally in myocardium tissue and is not detected as expected. In the WT mice injected with the pdes.C3, we could not detect the full-length protein in the heart but calpain3 cleavage fragments (60, 58 and 55 kDa) were observed. In contrast, no protein or cleaved products were detected in the heart of WT mice injected with pdes.C3-T. These results are compatible with a regulation occurring at translational level for the CAPN3 transgene bearing the miR208aT by the endogenous miR-208a, as observed in cellulo.

The miR-208a regulated pathway is not detectably disturbed by the miR-208a-regulated AAV vector

Because of the presence of the miR-208a target sequences downstream of the CAPN3 transgene, we were concerned about its potential titration effect towards the endogenous miR-208a, after transduction of pdes.C3-T vector in the heart. As a consequence, it might disrupt the regulation of its natural targets. We selected Hop and Cnx40 as the most potent biomarkers with respect to the modifications seen in the miR-208a knock-out animals. We monitored their mRNA levels together with the level of miR-208a in the hearts of mice (n=5 to 6) transduced with viral vectors.
carrying or not the miR-208a target sequences (pdes.C3 and pdes.C3-T). The results indicated that miR-208a, Cnx40 and Hop mRNA levels did not change significantly in the hearts of the WT mice transduced with pdes.C3-T compared to those injected with pdes.C3 (Figure 4E). We performed a power calculation to define the minimal difference that would have shown a statistical difference with the used sample size. For miR208, a significant difference would have been detected with values of 10 % of the mean of the control, of 50 % for Hop and of 75% for Cnx40. Taken together, these results gave no evidence that the presence of two miR-208aT sequences downstream of CAPN3 transgene could have interfered with the regulation of the two natural targets tested and have acted as “sponges” by titrating miR-208a in the heart.

No evidence of CAPN3 transgene toxicity in the liver

To evaluate the potential toxicity of the CAPN3 transgene vectors in the liver, the primary organ transduced following intravenous injection of AAV vectors, we injected WT and C3KO mice (n=3 to 4 per genotype) with AAV9-pdes.C3 (4.3x10^{13} vg/kg), monitored the blood level of the major hepatic enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and examined the liver histology at the end-point of the experiment. No histopathological sign was observed in the liver (Figure 5A). The level of ALT activity in the sera of the injected mice did not increase overtime compared to the control mice. Since ALT is mainly in the liver, the lack of increased level of this enzyme in the serum revealed an absence of hepatic cytolysis due to the CAPN3 transgene transfer (Figure 5B). The AST activity did not increase globally over the month in the injected mice except for one mouse that died prematurely and another one that showed strong sign of cardiac fibrosis after sacrifice (Figure 5B). AST is abundant in the liver but also in the cardiac and skeletal muscles. The absence of congruence seen on the graphs between ALT and AST for these two mice indicated that the increased AST level in their serum
could be associated to heart damage. Considering the expression level detected in cellulo in liver-of-origin cells transfected with plasmids carrying the CAPN3 and miR-206 promoters (Figure 3C), we also measured the blood level of ALT enzymatic activity over five weeks in animals (n=6) injected with 4.3x10^{13} vg/kg of pC3.C3 or p206.C3. No increased enzyme activity was observed in any of the injected animals compared to those injected with PBS (Figure 5C, p>0.05 for all points). Quantification of AAV vectors DNA (Figure 5D, left panel) and transgenic CAPN3 mRNA (Figure 5D, right panel) in the liver of mice injected with pdes.C3, pC3.C3 and p206.C3, revealed a similar level of transduction and expression with the different vectors. Despite the presence of messengers, the calpain3 protein was not detected in the liver after transduction of these transgenes (Figure 5E). Our data confirmed the absence of hepatic toxicity (Figure 5C) of these different AAV9 vectors tested (pdes.C3, pC3.C3 and p206.C3) which is not related to a lack of transduction (Figure 5D).

**Combinatorial arrangement of the muscle-specific promoters and miR208aT achieved desired expression profiles in cardiac and skeletal muscle**

We then constructed AAV vectors where the new CAPN3 and miR-206 promoters were associated with the target sequence of miR-208a (AAV2/9-pC3-CAPN3-miR208aT or pC3.C3-T and AAV2/9-p206-CAPN3-miR208aT or p206.C3-T). In vivo injection of these vectors plus pdes.C3-T in the tail vein of C3KO mice (n=4 to 5) were performed at the dose of 1.2x10^{13} vg/kg. All the mice were clinically monitored during the course of the experiment and sacrificed 3 months after injection. Anatomo-pathological examination of the heart indicated the absence of toxicity of all the three combined vectors as expected (Figure 6A). CAPN3 transgene expression from the different injected vectors was monitored by qRT-PCR. The presence of the miR208T sequence in the 3’UTR of the cassettes did not change the hierarchical order of expression of the
promoters that was previously observed in Figure 4C (Figure 6B). The calpain3 protein was not detected in the heart with any of these vectors (data not shown).

The expression of CAPN3 transgene was then monitored in skeletal muscles [tibialis anterior (TA) ; n=4 to 5 and psoas (PSO); n=3 to 5] and compared to the endogenous level of capn3 mRNA. The highest CAPN3 mRNA expression was observed with pdes.C3-T vector (31 and 21-fold higher in TA and PSO, respectively, compared to the endogenous level of capn3 mRNA; Figure 7A). The vector pC3.C3-T expresses 10 and 23% of the corresponding endogenous level of capn3 in the TA and PSO respectively, while these values are 40 and 220% for p206.C3-T (Figure 7A). The high calpain3 expression in skeletal muscles with pdes.C3-T was confirmed by western blot analysis. However, the presence of the protein is not detected above the background level with pC3.C3-T and p206.C3-T (Figure 7B). The immunostaining of TA muscles cross-sections confirmed the widespread expression of the transgenic calpain3 in the muscles of C3KO mice treated with pdes.C3-T (Figure 7C).

To analyze whether the expression of calpain3 observed in the muscle fibers had a positive effect on the pathological signs of the mouse model, we undertook an histological and morphological examination of the skeletal muscles of 4 weeks old C3KO (n=4 to 5) mice injected with pdes.C3-T, pC3.C3-T or p206.C3-T. The TA muscles injected with these vectors displayed improved histological features compared to those injected with PBS (Figure 8A). Morphometrical analysis of HPS-stained TA muscle sections showed a significant decrease of the number of centronucleated fibers in the muscles injected with the vectors compared to those injected with PBS (Figure 8B, left graph). Similar results were obtained with PSO muscles even if the decrease observed with pC3.C3-T and p206.C3-T is not statistically significant (Figure 8B, right graph). Altogether, these results indicate that the expression of the calpain3 in the
skeletal muscles transduced with our combined vectors can rescue the pathological features of the calpain3 deficient mice.

**Discussion**

In this report, we present experiments demonstrating the cardiac toxicity of *CAPN3* gene transfer with AAV vectors injected intravenously in mice. Our experiment indicates that the toxicity is strictly related to the proteolytic activity of the overexpressed calpain3 since no effect is seen with the C129S mutant. We previously demonstrated *in vitro* that transfection of calpain3 in non-muscle cells leads to cleavage of cytoskeletal proteins such as talin and subsequent collapse of the actin cytoskeleton leading to cell death. Interestingly, a conditional transgenic murine model overexpressing calpain3 specifically in the heart presents cardiac hypertrophy, reduced ejection performance and heart failure. This phenotype is highly similar to the one presented by transgenic overexpression of the ubiquitous calpain 1. It has been shown also that alterations in myocardial structure and function seen in cardiac pathologies are a consequences of cleavage of calpains substrates. Of note, among the cardiac proteins most sensitive to calpain 1 proteolysis are titin and α-fodrin, two known substrates of calpain3. All these data suggest that the mechanism of calpain3 cardiac toxicity could be due to an unregulated cleavage of substrates that would lead to lysis of transduced fibers and formation of a fibrotic scar.

Interestingly, no toxicity related to the transgenic expression of calpain3 was ever observed in skeletal muscle in any of our experiments. Similarly, a transgenic mouse model overexpressing at a high level calpain3 in skeletal muscle did not show any skeletal muscle impairment despite an up-regulation of 25 to 60-fold. These observations suggest a high buffering capacity of this tissue regarding the proteolytic activity of calpain3. It has been
proposed that titin, a gigantic sarcomeric protein carrying several calpain3 binding sites, serves as an inhibitory platform for the protease\(^{17-19}\). Interestingly, suppression of the activity of the protease through its interaction with titin has been demonstrated experimentally at the N2A region, a region of titin tissue-specific alternative splicing\(^{20,21}\).

The first strategy that we tested to prevent the cardiac toxicity was the introduction of miR-208a target sequences in the 3’UTR of \(CAPN3\) transgene. We validated the ability of miR-208a to block the expression of the transgene bearing the miR208a-target. Interestingly, our results strongly suggest a regulation at the translation level corresponding to the natural action mechanism of miR-208a\(^{12}\). In addition, we showed that the presence of the target sequences does not seem to exert titrating effect towards the endogenous miR-208a as its level was not affected and the two natural targets that we tested were not significantly deregulated \textit{in vivo} when the miR target bearing vectors were present. These results are in agreement with previous studies that have used miRNA-regulated vectors for fine-tuning of expression\(^{22-24}\). Moreover, further elements argue for the non toxicity of the approach. Indeed, silencing or genetic deletion of miR-208a are not associated with cardiac abnormalities but on the contrary are beneficial during heart failure\(^{10,25}\). A second strategy was to use skeletal muscle-specific promoters including the calpain3 promoter itself. In addition to prevent the cardiac toxicity, these promoters should reduce the possibility of toxicity in other organs. In particular, we showed no abnormalities in the liver, the primary organ transduced during AAV systemic injection. Interestingly, the systemic injection of the different vectors is accompanied by a decreased number of centronucleated muscle fibres in skeletal muscles albeit with variability according to the muscle. Considering the western blot results, it seems that even a low amount of calpain3 expressed in the skeletal muscles is sufficient to attenuate the phenotype of C3KO mice.
In conclusion, we demonstrated the potential toxicity of virally induced overexpression of calpain3 in the heart due to unregulated proteolytic activity. We were able to overcome the cardiac toxicity through modification of the cassette for restriction of CAPN3 transgene expression to the skeletal muscle while obtaining adequate expression for reversal of the pathological signs. Overall, our results highlight the complexity of gene replacement strategy and the care needed in selecting a strategy for gene replacement. We propose that the presented molecular strategies to prevent cardiac toxicity can not only be used for calpainopathies but also for any other transgene with potential toxicity in the heart.

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Conflict of Interest Disclosures: None.

References:


Figure Legends:

**Figure 1.** Cardiac toxicity of *CAPN3* transgene by systemic administration of AAV vectors. A/ Heart transverse sections from WT mice (n=4), 35 days after IV injection either with PBS or with 1.6x10^{13} vg/kg of the pdes.C3 or pdes.C3^{C129S}. Upper panels: HPS staining. Lower panels: Sirius red staining. Scale bars = 100 μm. B/ Representative image of whole heart transverse sections stained with Sirius Red. Scale bars = 500 μm. C/ Analysis of calpain3 expression by western blot in cardiac extracts from the injected WT mice with pdes.C3 and pdes.C3^{C129S}. Calpain3 is indicated by a black arrow.

**Figure 2.** Analysis of miR-208aT activity *in cellulo*. A/ Expression level of exogenous calpain3 (eYFP-C3) from the different constructs (Supplemental Figure 1B) was analyzed by western blot, 48 hours after transfection in HER911 cells (left panel). α-actin served as a loading control. Expression level of miR-208a in the cells was monitored by qRT-PCR (right panel). AU: arbitrary unit. Lane 1: non transfected, lane 2: eYFP-C3 + DsRed-β-actin, lane 3: eYFP-C3 + DsRed-premiR208a, lane 4: eYFP-C3-miR208aT + DsRed-β-actin, lane 5: eYFP-C3-miR208aT + DsRed-premiR208a. The experiment was performed 4 times. B/ Quantification of eYFP-C3 protein level normalized by α-actin protein level (left panel). Quantification of eYFP-C3 or eYFP-C3-miR208aT mRNAs level normalized by the respective plasmid quantity (right panel). The numbering of the samples is similar to A. AU: arbitrary unit. The experiment was performed 4 times.

**Figure 3.** Analysis of the *CAPN3* and miR-206 promoter activities *in cellulo*. A/. Activities of
CAPN3 (C3), miR-206 (miR206) and desmin (Desmin) promoters cloned upstream of the murine secreted alkaline phosphatase (mSeAP) reporter gene in transfected C2C12 myotubes. Alkaline phosphatase activity was measured in the supernatant from the day of differentiation initiation (D0) to the last day of differentiation (D5), and normalized with the plasmid quantity measured by qPCR. Non-transfected cells were used as negative control (NT). Error bars are smaller than the symbols on this figure. The experiment was performed twice. B/ Endogenous miR-206 and capn3 mRNA levels were monitored by qRT-PCR during C2C12 differentiation. AU: arbitrary unit. All experimental conditions gave a significant difference when comparing the initial point with the differentiation end point. The experiment was performed twice. C/ Activities of the CAPN3 and miR-206 promoters in diverse human cell types (n=3 to 4). The plasmids were transfected in different types of human cells (HER911, HUVEC, HT1080, HeLa, HuH-7, HEK293). Their promoting activities was monitored by mSeAP activity in the supernatant 48h later and normalized by the plasmid DNA quantity. Results obtained with the desmin, CAPN3 and miR-206 were compared to CMV and were found to be statistically significant but for the HUVEC cell line.

**Figure 4.** Analysis of the CAPN3 and miR-206 promoter activities in vivo. A/ Cardiac transverse sections from mice (n=6 but for pdes.C3-T n=3), 35 days after IV either with PBS or with identical doses (6x10^{12} vg/kg) of vectors pdes.C3, pdes.C3-T, pC3.C3 or p206.C3. The sections were stained with Sirius red (Upper panel, scale bar = 500μm) or HPS (lower panel), scale bar = 100μm. B/ Vector DNA levels in the heart of the injected WT mice were measured by qPCR. Each cross corresponds to one injected mouse. The mean for each condition is indicated by horizontal bars. C/ Trangenic CAPN3 mRNA level in the heart of the injected WT mice was
measured by qRT-PCR. The lane “H” refers to the level of the endogenous capn3 mRNA in a WT C57Bl/6 heart. Each cross corresponds to an injected mouse. The mean for each condition are indicated by horizontal bars. D/ Analysis of calpain3 expression by western blot in the skeletal muscle and heart of WT mice injected with PBS or the AAV vectors (pdes.C3 or pdes.C3-T). The calpain3 protein is indicated by a black arrow and its cleavage products (60, 58 and 55 kDa) by a bracket. MW: molecular weight. E/Quantification of miR-208a (miR208a), Hop and Cnx40 mRNAs levels in the heart of WT mice after IV injection either with pdes.C3 (n=6) or pdes.C3-T (n=5) at 6x10^{12} vg/kg. The miR208a data were normalized with sno202 and HOP and Cnx with P0. AU= Arbitrary Unit.

Figure 5. Analysis of CAPN3 gene transfer consequences in the heart and liver. A/ HE-stained liver cross sections of WT mice, 35 days after IV injection either with PBS (left panel) or with pdes.C3 (right panel) at 4.3x10^{13} vg/kg. Scale bars = 100 μm. B / ALT (left graph) and AST (right graph) assays were performed on sera from mice treated with PBS (open circles) or pdes.C3 (closed circles) at 4.3x10^{13} vg/kg. The sera were collected before the injection, 14 and 33 days post-injection. The activity was measured in international system unit per liter (U/L). Each circle corresponds to an injected mouse. The mean and SEM for each condition are indicated by horizontal and vertical bars, respectively. C / ALT assays were performed on sera from mice treated with PBS (open circles), pC3.C3 (left panel, closed circles) or p206.C3 (right panel, closed circles) at 4.3x10^{13} vg/kg (n=3 to 5). The sera were collected the day of injection, 7, 14, 21, 28 and 33 days post-injection. ALT activities are reported as in B. D/ Left panel: vector DNA levels in the liver of mice injected with PBS, pdes.C3 (n=4), pC3.C3 (n=3) and p206.C3 (n=3) at 4.3x10^{13} vg/kg, were measured by qPCR. Right panel: transgenic CAPN3
mRNA level in the liver of the injected mice was measured by qRT-PCR. The line “L” refers to the level of the endogenous *capn3* mRNA in a normal liver. Each cross corresponds to an injected mouse. The mean for each condition are indicated by horizontal bars. E/ Analysis of calpain3 level of expression by western blot in WT mice injected with PBS or the AAV vectors [pdes.C3, pC3.C3, p206.C3 or pdes.C3 C129S (C129S)]. The calpain3 protein is indicated by a black arrow and its cleavage products (60, 58 and 55 kDa) by a bracket. MW: molecular weight. GAPDH served as a loading control (grey arrow). No calpain3 protein was detected in the liver in the different conditions.

Figure 6. Prevention of CAPN3 transgene cardiac toxicity with the combined vectors. A/ Cardiac transverse sections from WT and C3KO mice, 4 months after IV injection either with PBS or with identical doses (1.2x10\(^{13}\) vg/kg) of the following vectors: pdes.C3 (n=4), pdes.C3-T (n=5), pC3.C3-T (n=5) and p206.C3-T (n=4). C3KO mice injected with pdes.C3 were used as controls to monitor CAPN3 transgene toxicity. They were sacrificed after one month in reason of the high toxicity of this vector. The image for this condition is framed to indicate the difference in timing. The cardiac sections were stained with Sirius red. Scale bar = 500μm. B/ Molecular analysis of the cardiac muscle. Left graph: quantification by qPCR of the vector DNA level in cardiac muscle of mice injected with the indicated AAV vectors. Right graph: quantification by qRTPCR of transgenic CAPN3 mRNA expressed in the heart of mice treated with the AAV vectors compared to the endogenous *capn3* mRNA level in WT mouse. The mice were injected at 4 weeks old and sacrificed 4 months after the injection (the n were identical to figure 6A). The lane “H” refers to the level of the endogenous *capn3* mRNA in a normal heart. Each cross corresponds to an injected mouse. The mean for each condition are indicated by horizontal bars.
**Figure 7.** *CAPN3* transfer efficiency with the combined vectors in skeletal muscle. A/

Quantification of *CAPN3* mRNA expressed in the TA (left graph) or PSO muscles (right graph) of C3KO mice injected intravenously with PBS or with the indicated vectors (pdes.C3; n=4 for TA and 3 for PSO, pdesC3-T, pC3.C3-T, p206.C3-T; n=5 for TA and PSO ) and sacrificed 4 months post-injection (1.2x10^{13} vg/kg each). The lane “Sk. M” refers to the level of the endogenous capn3 mRNA in a normal skeletal muscle. Each cross corresponds to an injected mouse. The mean for each condition are indicated by horizontal bars, B/

Analysis of calpain3 expression by western blot in the TA and PSO muscles from WT and C3KO mice injected with PBS or the indicated AAV vectors: pdes.C3C129S, pdes.C3-T, p206.C3-T. The calpain3 protein is indicated by a black arrow and its cleavage products (60, 58 and 55 kDa) by a bracket. α-actin served as a loading control (empty arrow). C/

Immunodetection of calpain3 in TA muscle.

Transverse sections of TA from WT mice injected with PBS and of C3KO mice treated either with PBS or pdes.C3-T were immuno-stained with an antibody directed against calpain3. Scale bar = 20μm. TA: *tibialis anterior* muscle, PSO: *Psoas* muscle.

**Figure 8.** Histological analysis of *CAPN3* gene transfer efficacy in skeletal muscles of calpain3-deficient mice. A/

Transverse sections of TA muscles from C3KO mice were stained with HPS, 4 months after IV injection either with PBS, pdes.C3-T, pC3.C3-T (n=5) or p206.C3-T (n=4) vectors (1.2x10^{13} vg/kg). Scale bars = 100 μm. B/

The number of centronucleated fibers (CNF/mm²) was measured in HPS-stained sections of TA (left graph) or PSO muscles (right graph) from C3KO mice injected either with PBS, pdes.C3-T, pC3.C3-T or p206.C3-T. The number of CNF is significantly different in TA and PSO muscles of C3KO mice injected with all these vectors compared to those injected with PBS except for pC3.C3-T in TA muscles and
p206.C3-T in PSO muscles. Difference with a $P$ value $< 0.05$ is indicated with an asterisk. TA: *tibialis anterior* muscle, PSO: *Psoas* muscle. Each cross corresponds to an injected mouse. The mean for each condition are indicated by horizontal bars.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 6
Figure 7
Figure 8
Restriction of Calpain3 Expression to the Skeletal Muscle Prevents Cardiac Toxicity and Corrects Pathology in a Murine Model of Limb-Girdle Muscular Dystrophy
Carinne Roudaut, Florence Le Roy, Laurence Suel, Jerome Poupiot, Karine Charton, Marc Bartoli and Isabelle Richard

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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Constructions of vectors

To clone the \textit{CAPN3} promoter, a DNA fragment corresponding to the 5'-flanking region of the human \textit{CAPN3} gene with a size of 1.65 kb (from -1651 to -1) was amplified by PCR using a human DNA clone (BAC RCP11-164J13, Genbank n° ACO12651, a gift from Dr. Roland Heilig, Centre National de Séquençage, Evry, France) and cloned in a \textit{mSeAP} plasmid (previously described in \cite{1}) upstream of the reporter coding sequence in replacement to the original promoter to give the plasmids pAAV-pC3\_1650-mSeAP. To clone the \textit{miR-206} promoter, a DNA fragment (pmiR206: 817 bp) of the 5' flanking region of the human \textit{miR-206} was synthesized (Genecust, EUROPE LaBBX, Luxembourg) and cloned in the pAAV-desmin-mSeAP plasmid in replacement of the desmin promoter. The sequence of the human microRNA 208a (\textit{miR-208a}) was retrieved from the miRBase (MI0000251) and a fragment composed of tandem copies of the \textit{miR-208a} target sequence (\textit{miR208aT}: ACAAGCTTTTGTGCCTCCTTAT) was synthesized (Eurofins, Les Ulis, France). The \textit{miR208aT} was introduced downstream of a cassette expressing a protein composed of eYFP fused to a C129S mutated murine calpain-3 to obtain pCMV-eYFP\textit{capn3}\textsuperscript{C129S}\textit{-miR208aT}. A DNA fragment of 538bp containing the premiR-208a (located in intron 27 of \textit{α-MHC}) was retrieved by PCR from human genomic DNA and cloned downstream of the DsRed coding sequence in pCMV-DsRed-\textit{βactin} plasmid (a gift of Dr. Jeanson, Genethon, Evry, France) in replacement of \textit{β–actin} to obtain pCMV-DsRed-premiR208a.

The pdesm.CAPN3 plasmid was derived from pC5-12.CAPN3 \cite{2} by replacing the C5-12 promoter with the desmin promoter \cite{3}. The pdesm.CAPN3\textsuperscript{C129S} was derived from a pcDNA3
plasmid previously described by cloning into an AAV vector. The 1.65 kb DNA fragment of the human \textit{CAPN3} promoter was cloned in pdesm.CAPN3 in replacement of the desmin promoter to obtain the plasmid pC3.CAPN3. The pmiR206 fragment was cloned in pdesm.CAPN3 to obtain the plasmid pmiR206.CAPN3. The target sequence miR208aT was cloned in pdesm.CAPN3 or in pmiR206.CAPN3 to obtain pC3.CAPN3-miR208aT and pmiR206.CAPN3-miR208aT.

The premiR-208a containing fragment was fused downstream of the red fluorescent protein (DsRed) cDNA (DsRed-premiR208a), to monitor its effective transfection into the cells, and the \textit{DsRed} cDNA fused to \textit{\beta}-actin cDNA (DsRed-\textit{\beta}-actin) was used as a negative control (Supplemental Figure 1B). The \textit{capn3} plasmids are coding for a fusion protein composed of the enhanced yellow fluorescent protein (eYFP) and the murine calpain 3 mutated on its catalytic site (C129S) with and without the miR208aT sequence downstream (eYFP-C3 and eYFP-C3-miR208aT, Supplemental Figure 1B).

**Interspecies analysis of \textit{CAPN3} and miR-206 promoters**

As a first step to clone the human \textit{CAPN3} and miR-206 promoters, we performed an interspecies comparative analysis of the sequences upstream of the \textit{CAPN3} gene and miR-206 using the Evolutionary Conserved Region web platform (ECR; http://ecrbrowser.dcode.org/). All mammalian sequences available were used to identify the regions of highest homologies with the Human sequence (Supplemental Figures 2 and 3). Concerning \textit{CAPN3}, a first region of about 510 bp that encompasses the 5’ UTR region and the sequence upstream of the transcriptional start at - 310 presents a homology higher than 70\% in all the species (Supplemental Figure 2; inset 1). A second and third (around 120 and 340 bp) islands of highly significant homology (65 \% across all the species) was identified 0.41 and 1.10 kb, respectively, upstream of the transcription start (Supplemental Figure 2; insets 2 and 3).
Looking at the human/mouse conserved transcription factor binding sites computed with the Transfac Matrix database (v7.0; http://www.gene-regulation.com/pub/databases.html) identified binding sites for a number of transcription factors involved in muscle processes such as myocyte enhancer factor 2 (MEF2; Supplemental Figure 2).

For miR-206, it was previously published that the mouse miR-206 promoter presents two cis-regulatory modules (CRM): one immediately upstream to the miRNA sequence and the second 800 bp further upstream. Both regions are conserved among mammalian species and carry muscle specific transcription factor binding sites such as MyoD or myogenin (ECR web platform, Supplemental Figure 3A).

**Analysis of transcriptional activities of the CAPN3 and miR-206 promoters on different cell lines**

To ascertain their selectivity for muscle, the CAPN3 and miR-206 promoters activity were studied in different human cell lines: Human embryonic Retinoblasts (HER911), Human umbilical vein-endothelial cells (HUVEC), Human fibrosarcoma cells (HT1080), Human cervix adenocarcinoma cells (HeLa) and Human hepatocellular carcinoma cells (HuH-7). Plasmids with the Desmin and CMV promoters were also used in this experiment for comparison. No reporter activity was detected in the different cell lines for the both promoter fragments with the exception of HuH-7 cells (Figure 3B). To define whether this expression was specific to this particular cell line or whether the cloned fragments were able to drive expression in liver-of-origin cells, a second analysis was performed using the hepatocellular carcinoma HepG2 cell line. A similar result was obtained with this cell line (data not shown).

We performed an analysis of the endogenous expression of CAPN3 mRNA and miR-206 in all the cell lines. No statistically significant difference was observed between the hepatic cell lines and the others regarding these two genetic entities (data not shown), indicating no
particular liver expression of both miR-206 and calpain 3. The observed discrepancy between the endogenous and the cloned promoters could be related to the absence a repressive element in the cloned fragments or else that the liver cell lines are more permissive for the cloned promoters.

**Quantitative PCR**

For quantification of endogenous microRNA expression, 20 ng of total RNA extraction was reverse transcribed using the “TaqMan MicroRNA Reverse transcription” kit and subjected to the MicroRNA Assays (Applied Biosystems). Normalization across samples was performed with the expression of snoRNA202.

The following Taqman MicroRNA assays (Applied Biosystems) were used for quantitative PCR of microRNA: assay ID511 for miR-208a, assay ID510 for miR-206 and assay ID1232 for snoRNA202 (normalization across samples).

For other quantifications, one µg of total RNA was reverse-transcribed using random hexamers and oligodT and the Verso cDNA kit (Abgene) or the RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas). Real-time PCR was performed using ABI-PRISM7700 system (Applied Biosystems). For calpain 3 analysis, serial standard dilution of the same control RNA was used in each experiment and processed along with the experimental samples to bypass the variability in the efficiency of the cDNA preparation and the PCR and to be able to compare different experiments. This RNA was prepared by an in vitro transcription reaction from a plasmid carrying a calpain 3 cDNA mutated to accommodate all the primer pairs. The qRT-PCR results were expressed in arbitrary unit as a ratio with the P0 gene. The expression results from the analysis of transfected cells were normalized by the quantification of plasmid DNA extracted using DNeasy Blood and tissue kit (Qiagen). DNA quantification was performed using primer pairs and TaqMan-MGB
probes specific to the ITR sequence and proceed by Sybergreen qPCR using 10 ng of DNA. The expression results from the analysis of transfected cells were normalized by the quantification of plasmid DNA extracted using DNeasy Blood and tissue kit (Qiagen).

The following TaqMan® Gene Expression Assays (Applied Biosystems) were used: Gja-5 [Mus musculus]: Mm00433619_s1 for connexin 40, HOP homeobox [Mus musculus]: Mm00558630_m1 for Hop and Mm00482985_m1 (targeting the exon 11-exon 12 junction) for murine Capn3. The following primer pairs and Taqman probe were used: [CAPN3sfr.f, 5’_CGCCTCCAAGGCCCCGT_3’; CAPN3sfr.r, 5’_GGCGGAAGCGCTGGCT_3’; and MGBTUCAPN3.p, 5’_CTACATCAACATGAGAGGT_3’] for the human transgene calpain 3, [CAPN3.f 5’_CGCCTCCAAGGCCAGG_3’, CAPN3.r 5’ GGCGGAAGCGCTGGGA_3’ and CAPN3.p 5’_TACATCAACATGCGGGAGGT_3’] for the human calpain 3 (for analysis of C129S) and [MH181PO.F: 5’_CTCCAAGCAGATGCAGAGA_3’, M267PO.R: 5’_ACCATGATGCAGCAAGATGA_3’, M225PO.p: 5’_CCGTGGTGCTGATGGGCAAGA_3’] for the ubiquitous acid ribosomal phosphoprotein gene P0 (P0).

In C2C12, the DNA quantification was proceed by Sybergreen qPCR using 10 ng of DNA and the primer pairs located in the β-globin intron of the AAV cassette: Intron3.fw, 5’_CAAGACAGGTTTAAGGAGACCAATAG_3’; Intron4.rev: 5’_GTAAGACCAATAGGCTATCAAGA_3’. For human cells and muscles, the DNA quantification was performed on 100 ng of DNA using primer pairs and TaqMan MGB probes specific to the ITR sequence as follows: 1AAV65/Fwd: 5’_CTCCATCACTAGGGTTTCCTTGTA_3’, 64AAV65/rev: 5’_TGGCTACGTAGATAAGTAGCATGGC_3’ and AAV65MGB/taq: 5’_GTTAATGATTAACC_3’.
In vivo experiments

For intravenous injection (IV), the experiments were performed with the highest doses possible with respect to the titers of viral preparations and to ensure an equivalent dose of each vector for the purpose of comparison. In the initial experiments with the different serotypes, male animals were injected with the highest dose possible according to the viral preparations obtained (AAV1: $1.6 \times 10^{13}$, AAV8: $7.0 \times 10^{12}$ and AAV9: $4.3 \times 10^{13}$ vg/kg) plus for AAV9 with a series of dilutions ($4.0 \times 10^{12}$, $1.0 \times 10^{13}$ or $1.6 \times 10^{13}$ vg/kg). The control experiment with the AAV-C129S was performed at the dose of $1.6 \times 10^{13}$ vg/kg. In the other experiments, male mice were injected with doses of $6 \times 10^{12}$ vg/kg for the comparing AAV9-pdes.C3, AAV9-pdes.C3-T, AAV9-pC3.C3 and AAV9-p206.C3, $1.2 \times 10^{13}$ vg/kg for comparing AAV9-pdes.C3, AAV9-pdes.C3-T, AAV9-pC3.C3-T and AAV9-p206.C3-T and $4.3 \times 10^{13}$ vg/kg for evaluating liver toxicity. The injections were done in a 800µl volume of the viral preparation into the tail vein and as control, C57Bl/6 and C3KO mice were injected with 800µl of PBS. All the in vivo experiments were performed at the age of 8 weeks except for the last one aimed at evaluation of the reversal of the pathological signs that was performed at the age of 4 weeks, therefore before the onset of the disease. In that case, the injected volume was reduced to 280 µl. Blood samples were collected without anticoagulant every two weeks. Sera were analyzed using the VITROS DT60 apparatus (Ortho Clinical Diagnostics, UK) using Vitros ALT and AST DT slides for determination of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities. Mice were sacrificed five weeks ($n= 4$ and 6 for all experiments but the functional evaluation presented in figure 8) or four months ($n= 5$ for the experiment presented in figure 8) after injection.
**SUPPLEMENTAL TABLE A**

Table: Outcome of the intravenous injections of pdes.C3

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SUPPLEMENTAL FIGURES

Supplemental Figure 1: miR-208T activity in cellulo. A/ Expression level of miR-208a in the heart, liver, tibialis anterior (TA) and soleus (SOL) skeletal muscles, from WT and C3KO mice, measured by qRT-PCR and normalized with snoRNA202. The results confirm the cardiac specificity of miR-208a and the absence of deregulation of its expression following calpain-3 deficiency. B/ Schematic representation of the constructs made to study the efficiency of the miR-208a target sequence in cellulo. CMV: cytomegalovirus promoter; DsRed: red fluorescent protein; eYFP: enhanced yellow fluorescent protein; capn3\textsuperscript{C129S}: inactive form of murine calpain 3; 2xmiR208aT: target sequences of miR-208a in tandem; premiR-208a: β-MHC intronic sequence containing the premiR-208a sequence.
**Supplemental Figure 2: CAPN3 promoter.** ECR Browser conservation profile of the Human genomic region upstream of the CAPN3 gene (hg19: chr15:42650353-42652003) compared to the other mammalian species available. Numbering is relative to the ATG. The boxes indicate the three conserved regions across species. The 5’untranslated region (5’UTR) of the CAPN3 gene is in light green, the conserved intergenic regions in red and repetitive elements in dark green. The main conserved transcription factor binding sites (http://rvista.dcode.org/) are indicated by black arrows below the alignment and commented in the insets below. The DNA fragment tested in our study (pC3) is represented by a black bar on top of the scheme.

![Supplemental Figure 2: CAPN3 promoter](image-url)

- **GR**: glucocorticoid receptor (muscle atrophy)
- **HSF1/2**: heat shock transcription factor 1 and 2 (regrowth of skeletal muscle)
- **MEF2**: myocyte enhancer factor-2 (myogenesis regulation)
- **E2F**: key regulator of cell proliferation
- **ATF4**: activating transcription factor 4 (promotes skeletal muscle atrophy during fasting)
- **P300**: muscle-specific transcription factor
- **COUP**: chicken ovalbumin upstream promoter (skeletal muscle differentiation)
- **TCF4**: transcription factor 4 (myogenesis regulation)
**Supplemental Figure 3: MiR-206 and its promoter.**

**A/** miR-206 promoter. ECR Browser conservation profile of the Human genomic region upstream of miR-206 (hg19, chr6: 52008028-52009228) compared to the other mammalian species available. Numbering is relative to the miRNA sequence. The two boxes indicate the conserved regions across species. The premiR-206 sequence is in light green, the conserved regions are in red and repetitive elements in dark green. The main conserved transcription binding sites are indicated by black arrows below the alignment and commented in the insets below. The construct tested in our study (pmiR206) is represented by a black bar on top of the scheme.

**B/** The expression level of miR-206 was measured by qRT-PCR in skeletal and cardiac muscles from WT and C3KO mice (normalization with snoRNA202). TA: *tibialis anterior* muscle. AU: arbitrary unit. The result confirms the muscle specificity of miR-206 and the absence of deregulation of its expression following calpain-3 deficiency (C3KO mice).
SUPPLEMENTAL REFERENCES


