INTRODUCTION

Many diseases (neuromuscular or chronic inflammatory diseases, cancer...) are associated with skeletal muscle atrophy. Muscle wasting occurs also as a natural process of aging and can lead to sarcopenia, a generalized loss of muscle mass and function. These muscle tissue defects are highly disabling for patients, especially since there is a lack of adequate treatments.

GASP-2 overexpressing mice exhibit a hypermuscular phenotype with contrasting molecular effects compared to GASP-1 transgenics

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Abstract

Muscle atrophy is associated with many diseases including genetic disorders, sarcopenia, or cachexia syndromes. Myostatin (Mstn), a transforming growth factor-beta (TGF-β) member, plays a key role in skeletal muscle homeostasis as a powerful negative regulator. Over the last decade, about 15 clinical trials aimed at inhibiting the Mstn pathway, failed to produce conclusive results. In this context, we investigated whether growth and differentiation factor-associated serum protein-1 (GASP-1) or GASP-2, two natural inhibitors of Mstn, might represent a potential therapeutic. As we previously reported, mice overexpressing Gasp-1 (Tg(Gasp-1)) present an increase of muscle mass but develop metabolic disorders with aging. Here, we showed that overexpression of Gasp-2 increases the muscular mass without metabolic defects. We also found that Tg(Gasp-2) mice displayed, like Mstn⁻/⁻ mice, a switch from slow- to fast-twitch myofibers whereas Tg(Gasp-1) mice exhibit a reverse switch. Our studies supported the fact that GASP-2 has less affinity than GASP-1 for Mstn, leading to a constitutive Mstn upregulation only in Tg(Gasp-1) mice, responsible for the observed phenotypic differences. Altogether, our findings highlighted a gene expression regulatory network of TGF-β members and their inhibitors in muscle.

KEYWORDS

muscle, myostatin, GASP-1, GASP-2, hypertrophy, hyperplasia

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treatments. Improving our understanding of the mechanisms responsible for skeletal muscle atrophy in patients is important in order to develop therapies to prevent these clinical conditions.

Skeletal muscle is composed of heterogeneous muscle fibers bundled together and which differ in their metabolism and contractile properties. This type of organization confers to skeletal muscle remarkable levels of plasticity in the face of changes to the external environment. During embryonic development, the number and the size of myofibers increase (hyperplastic and hypertrophic growth) until birth. Postnatal muscle growth is then only achieved by myofibers hypertrophy and can be divided into two distinct steps. Between birth and weaning in mouse, hypertrophy is supported by a rapid increase of the nuclei number within myofibers via the activation and fusion of satellite cells. From 3 weeks old to adulthood, muscle mass regulation is dependent of a balance between protein synthesis and degradation. This protein turnover is induced in response to various stimuli such as exercise, inactivity, or environmental factors (hypoxia, heat, nutrient availability, and growth factors). During the past two decades, much progress has been made in unraveling the molecular mechanisms underlying either adult muscular hypertrophy or atrophy.

Myostatin (Mstn), a member of the transforming growth factor-beta (TGF-β) superfamily, is an important negative regulator of skeletal muscle growth, homeostasis and repair. Myostatin knockout (Mstn−/−) mice exhibit at 3 months an increase in muscle mass due to both hyperplasia and hypertrophy of myofibers. Mstn−/− null mice have reduced body fat and increased tolerance to glucose, protecting them from age-related obesity. Targeting the Mstn signaling pathway may offer promising therapeutic strategies for the treatment of muscle wasting disorders. Although several clinical trials by inhibiting Mstn are conducted, the first results are controversial except in a gene therapy trial based on the inhibition of Mstn by follistatin, one of its natural inhibitors. In this context, we investigated whether the paralogs growth and differentiation factor-associated serum protein-1 (GASP-1) and GASP-2, two other natural Mstn inhibitors, might represent a potential therapeutic.

Generation of transgenic lines overexpressing Gasp-2

The 1656 bp coding sequence of the murine Gasp-2 gene was amplified by primers 5'-ATGCTTGGGACAGCCATTC-3' and 5'-GTATTGGAACCGTGAGCAGTTC-3' (transcript sequence ENSEMBL ENSMUSG-00000071192) and was introduced into the expression vector pCDNA3.1/V5-His TOPO (Invitrogen) where Gasp-2 cDNA is under the cytomegalovirus (CMV) promoter/enhancer. A purified Sal1-NsiI fragment was microinjected into the male pronucleus of musculus spermatozoa. Male pronucleus injected embryos were subsequently transferred into the oviducts of CD1 pseudopregnant females and confirmed by PCR using primers 5'-ATGCCTGCCCCACAGCCATTC-3'.

The generated transgenic lines Tg(Gasp-1) and Tg(Gasp-2) overexpressing GDF-11, Mstn−/−, and control animals are on FVB/N background. All mice were bred and housed in the animal facility of Limoges University under controlled conditions (20°C, 12 hours light/12 hours dark cycle) with free access to standard mouse chow and tap water. Experimental procedures were carried out in accordance with European legislation on animal experimentation (Directive 2010/63/UE) and approved by the ethical committee n°033 (APAFIS #1903-2015091612088147 v2).

Phenotypic and molecular analyses were performed on 3-weeks-old, 3-month-old, and 16-month-old mice, independently of animal sex.

2.2 | Generation of transgenic lines overexpressing Gasp-2

METHODS

Animals

Myostatin deficient mice (Mstn−/−) and Gasp-1 overexpressing mice (Tg(Gasp-1)) have been described previously. The generated transgenic lines Tg(Gasp-2) overexpressing Gast-2, Mstn−/−, Tg(Gasp-1), and control animals are on FVB/N background. All mice were bred and housed in the animal facility of Limoges University under controlled conditions (20°C, 12 hours light/12 hours dark cycle) with free access to standard mouse chow and tap water. Experimental procedures were carried out in accordance with European legislation on animal experimentation (Directive 2010/63/UE) and approved by the ethical committee n°033 (APAFIS #1903-2015091612088147 v2).

Phenotypic and molecular analyses were performed on 3-weeks-old, 3-month-old, and 16-month-old mice, independently of animal sex.
of one-cell fertilized FVB/N embryos. Two independent homozygous lines overexpressing Gasp-2, named Tg(Gasp-2.2), and Tg(Gasp-2.9), were obtained from two different founders and were characterized.

2.3 Copy number genotyping

Copy number genotyping was done using SYBR Green-based Real-Time PCR from Tg(Gasp-2) genomic DNA (QuantStudio 3 system, ThermoFisher Scientific). To determine the average inserted Gasp-2 transgene number, we used C-C chemokine receptor type 5 (Ccr5) as endogenous reference gene to normalize the amount of chromosomal DNA (number of transgene copy number by cell = 2 ΔCt X2 – 2). PCR assays were carried out as previously described in Monestier et al using the following primers: Gasp-2-Fwd (5’-ATGCACGTGACGACCTGTG-3’) and Gasp-2-Rev (5’-CTGCTCTCGAGTGTGGCCCG-3’) primers targeting Gasp-2 exon 2; Ccr5-Fwd (5’-GCACCAAGAGACCTTGGGAGCA-3’) and Ccr5-Rev (5’-GTCTAGTCTGACCACAGCA-3’) primers targeting Ccr5 exon 2. Data were analyzed by the QuantStudio Design & Analysis software.

2.4 RNA extraction, retrotranscription and gene expression analysis

Total RNA from tissues, cells, or embryos were isolated using RNeasy midi kit (Qiagen). Synthesis of cDNA was performed with the High Capacity cDNA Archive kit (Applied Biosystems) to convert 2 μg of total RNA into single-stranded cDNA. Taqman copy number assays were done with Gene Expression Master Mix (Applied Biosystems, Waltham, MA, USA), according to the manufacturer’s instructions. Twenty nanograms of cDNA were run in triplicate on QuantStudio 3 real-time PCR system (Applied Biosystems) with Taqman primers and probe sets: 18S (Hs99999901_s1), glyceraldehyde-3-phosphate dehydrogenase (Gapdh) (Mm99999915_g1), beta-2-microglobulin (β2m) (Mm00437762_m1), Gasp-1 (Mm00725281_m1), Gasp-2 (Mm01308311_m1), Mstn (Mm03024050_m1), Myogenic regulatory factor 4 (Mrf-4) (Mm00435126_m1), and Myog (Mm00446194_m1). Relative mRNA expression values were calculated by the ΔΔCt method with normalization of each sample to the average change in cycle threshold value of the controls.

TaqMan low-density array (TLDA, Applied Biosystems) assays were performed based on the same above conditions, except that 200 ng cDNA were used per TLDA card. TLDA cards present 43 selected genes involved in TGF-β signaling pathway as previously described.

2.5 Enzyme-linked immunosorbent assay (ELISA) of GASP-2

Growth and differentiation factor-associated serum protein-2 concentration from mouse plasma was determined in a sandwich ELISA according to the manufacturer’s instructions (GASP-2/WFIKKN DuoSet ELISA, R&D Systems). All measurements were performed in triplicate and data for the standard curve were fitted to a logistic plot with the MARS Data Analysis Software (BMG Labtech) to determine the levels of GASP-2.

2.6 Protein extraction and immunoblotting

Total cell protein extracts were prepared from frozen tissues or cell pellets, solubilized for 2 hours at 4°C in a RIPA lysis buffer (50 mM Tris, pH 8, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, and protease inhibitors). Protein lysates were centrifuged at 12 000 g for 20 minutes at 4°C, and protein supernatant concentration was determined at A595 nm using the Bradford assay (Bio-Rad). Equal amounts of proteins (50 μg) were resolved by SDS-PAGE using 10% polyacrylamide gels and then transferred onto Amersham Protran premium 0.2 μm nitrocellulose (GE Healthcare, Buckinghamshire, UK). Membranes were blocked using 5% nonfat dry milk (w/v) in TBST 0.1% buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4, 0.1% Tween-20) for 1 hour at room temperature. Specific primary antibodies were diluted in 2.5% nonfat dry milk and incubated overnight at 4°C: anti-phospho-Sma Mothers Against Decapentaplegic homolog (Smad2/3) (polyclonal Rabbit 1:500, AB3225, R&D Systems), anti-Smad2/3 (Polyclonal Goat 1:500, AF3797, R&D Systems), anti-V5 (monoclonal mouse 1:1000, MA5-15253, Invitrogen) and anti-mouse GAPDH antibody (Goat polyclonal 1:2000, AF5718, R&D Systems). After three washes with TBST, membranes were incubated with secondary antibodies (anti-goat, anti-rabbit or anti-mouse HRP-conjugated IgG, Dako, Glostrup, Denmark) diluted at 1:1000 in TBST, 2.5% (w/v) nonfat dry milk for 1 hour at room temperature. After three washes in TBST, reactive proteins were visualized with ECL Prime Western blotting system (GE Healthcare, Uppsala, Sweden). For detection and relative quantification of band intensities, we used Amersham Imager 600 device (GE Healthcare).

2.7 Immunofluorescence staining

Skeletal muscles having a glycolytic, oxidative or mix metabolism (tibialis anterior, gastrocnemius, flexor digitorum longus, and soleus) were frozen in liquid nitrogen-cooled isopentane, stored at −80°C and sectioned (10 μm thick). Cryosections were
thawed at room temperature and air-dried. A permeabilization step was required only for paired box 7 (Pax7) staining with cold methanol at −20°C and a treatment for antigen retrieval in 10 mM citrate buffer, pH 6 at 90°C for 2 × 5 min. Then, cryosections were blocked for 1 hour at room temperature in blocking buffer (5% BSA in phosphate buffered saline (PBS)) or (10% goat serum, 1% bovine serum albumin (BSA), and 0.1% Triton X-100 in PBS) for Pax7 staining. Incubation with primary antibodies diluted in BSA 1%/PBS took place overnight at 4°C for Pax7 staining and 1 hour at 37°C for other staining. Primary antibodies used for these analyses were anti-laminin (Rabbit IgG, 1/500, L9393, Sigma-Aldrich), anti-Pax7 (Mouse IgG1, 1/100, MAB1675, R&D System), and different anti-myosin: BA-D5 for Type I (Mouse IgG2b, 1/3, Agro-bio), SC-71 for Type I (Mouse IgG1, 1/3, Agro-bio), BF-F3 for type Ib (Mouse IgM, 1/3, Agro-bio), and 6H1 for Type Ix (Mouse IgM, 1/100, DSHB). After washes, slides were incubated for 30 minutes at 37°C with DAPI (1/1000) and secondary antibodies conjugated to a fluorescent dye diluted in BSA 1%/PBS and secondary antibodies conjugated to a fluorescent dye diluted in BSA 1%/PBS: AlexaFluor-350 Goat Anti-Mouse IgG2b, Alexa-Fluor-546 Goat Anti-Mouse IgG1, Alexa-Fluor-488 Goat Anti-Mouse IgM, or Alexa-Fluor-633 Goat Anti-Rabbit IgG (Invitrogen). After washes, the slides are mounted with a coverslip with Mowiol solution and colorless varnish. Scan of the entire muscle area were acquired with an automated Nikon inverted epifluorescence microscope with NIS Element Software. Myofiber area and number were calculated semiautomatically from laminin-stained cryosections using ImageJ software in whole muscle cross sections. Total and Pax7+ myonuclei were automatically counted using ImageJ software. Fiber typing was performed as previously described.23 Briefly, the fiber type characterization was realized by semiautomatic image analysis Visilog software (FEI), using the double laminin/myosin labelling.

2.8 | Isolation of satellite cell-derived myoblasts and cell culture

Primary myoblasts were obtained from 5-weeks-old male wild-type (WT) or Tg(Gasp-2.9) mice. Briefly, murine myoblasts were isolated from hindlimb muscles after enzymatic digestion by pronase (Sigma-Aldrich, P-5147) diluted in Ham’s-F10 medium (Gibco) and 1% penicillin/streptomycin, 1 hour at 37°C. The solution was centrifuged for 5 minutes at 800 rpm to remove undigested fragments. The supernatant was filtered on 45 μm cell strainers. The cells are washed three times in Ham’s-F-10 medium and centrifuged at 1500 rpm for 20 minutes at room temperature. Mouse satellite cells are isolated by depletion of nontarget cells using the Satellite Cell Isolation Kit (Miltenyi Biotec). Cells were plated on Matrigel-coated Petri dishes (BD Biosciences) in Growth Medium (GM); Ham’s-F10 supplemented with 20% horse serum and 1% penicillin/streptomycin supplemented with 5 ng/mL basic fibroblast growth factor (bFGF, Invitrogen). Cells were maintained at 37°C in a water-saturated atmosphere containing 5% CO2 in air. To induce differentiation, primary myoblasts at 80% confluence were placed in Differentiation Medium (DM) consisting of Ham’s F10 with 10% horse serum and 1% penicillin/streptomycin.

2.9 | Proliferation assay and fusion index measurement

Primary myoblast proliferation was assessed as described in Oliver et al.24 Cells were seeded at 2500 cells per well in GM in 96-well microtiter plates and fixed at regular 24 hours periods before methylene blue staining and measured at A590nm using an ELISA plate reader (FLUOstar Omega; BMGLabtech, Ortenberg, Germany). Point 0 hour of proliferation does not correspond to plating but to 6 hours post-plating. Fusion index measurement was performed by immunofluorescence as previously described.25 Cells were fixed in 4% paraformaldehyde for 10 minutes and permeabilized with 0.1% Triton X-100PBS for 30 minutes at 4°C. The cells were washed three times in PBS 1X and saturated for 1 hour at room temperature using PBS with 20% goat serum. Then, the cells were stained with the primary antibody 1:500 in PBS-BSA 4% (Anti-MyHC Class II antibody, Abcam) overnight in a humid atmosphere at 4°C. Cells were washed three times for 5 minutes with 0.01% Tween 20-PBS and incubated with the Alexa-Fluor conjugated secondary antibody (1:1000) and DAPI (1:1000) in PBS-BSA 4% for 15 minutes at 37°C in a humid atmosphere. Images were acquired with a Leica DMI6000B inverted epifluorescence microscope using the MetaMorph software (Molecular Devices, Sunnyvale, USA). Fusion index was calculated by dividing the number of myonuclei contained in MyHC-expressing myotubes by the total number of myonuclei (ImageJ software).

2.10 | Metabolic analyses

For intraperitoneal glucose tolerance test (IPGTT), 16 hours-fasted mice were injected with 20% D-glucose (2 mg/g body weight). Glucose levels were measured using a glucose meter (OneTouch Ultra) from tail blood at 0, 15, 30, 60, and 120 minutes after glucose injection.

2.11 | Skeletal muscle enzymatic activities

Lactate dehydrogenase (LDH) and isocitrate dehydrogenase (ICDH) were measured from a 5% (w/v) muscle (quadriceps or gastrocnemius) homogenate in pH 8.0 buffer (250 mM sucrose, 2 mM EDTA, 10 mM Tris). The frozen muscle was crushed in the buffer on ice with an ultra-turax. After centrifugation at 6000 rpm for 15 minutes at 4°C, the supernatants were removed.
and stored on ice until the enzymatic activities were measured using the Konelab 30 controller (Thermo Scientific). The measurement of the LDH and ICDH activities at A₃₄₀nm was based on the NADH disappearance or production, respectively. The reactions were done in LDH buffer (Triethanolamine 50 mM/EDTA 5 mM/Pyruvate de sodium 2 mM/NADH 0.234 mM/pH 7.5) or ICDH buffer (Na₂HPO₄ 36.1 mM/MgCl₂ 0.5 mM/Triton 0.05%/NADP 0.334 mM/Isocitrate 1.29 mM/pH 7.3)

2.12 | Statistical analyses

Unless otherwise stated, results are expressed as mean ± SEM. One-way ANOVA was performed to examine the effect of genotype (WT vs genotype) on each parameter. Statistical significance was set at P < .05. A minimum of three replicates were performed for each experimental condition.

3 | RESULTS

3.1 | Generation of Gasp-2 transgenic mouse lines

We constructed a transgene expressing mouse Gasp-2 cDNA under the control of a CMV promoter to create mice overexpressing ubiquitously Gasp-2. Two independent Tg(Gasp-2) lines were successfully established and named Tg(Gasp-2.2) and Tg(Gasp-2.9). Transgene copy number was estimated by semi quantitative real-time PCR using Ccr5 gene as an endogenous reference to normalize the amount of chromosomal

![Figure 1](image_url)
DNA. The homozygous Tg(Gasp-2.2) mice harbored ~ six copies of the transgene, while the Tg(Gasp-2.9) mice had ~ four copies (Figure 1A). The copy number was stable within all subsequent generations. The two Tg(Gasp-2) lines displayed a strong expression of Gasp-2 (100- to 10 000-fold compared to WT) in various tissues (Figure 1B) and muscles (Figure 1C). The transgene-driven GASP-2 protein expression was further analyzed by western blotting with an anti-V5 antibody (Figure 1D), confirming the GASP-2 overexpression in both lines. As GASP-2 is a secreted protein, we measured its amount in serum and showed a 3-fold overexpression of GASP-2 in Tg(Gasp 2.2) mice and 4-fold in Tg(Gasp 2.9) mice (Figure 1E).

3.2 Overexpression of Gasp-2 leads to a hypermuscular phenotype due to hypertrophy without hyperplasia

Mice overexpressing Gasp-2 have a higher overall weight compared to WT mice from weaning to 90 days (Figure 2A). Compared to 3-month-old WT mice, the Tg(Gasp-2.2) and Tg(Gasp-2.9) animals exhibit a total body weight increase of 11.5% and 13%, respectively. Furthermore, the overexpression of Gasp-1, the paralog of Gasp-2, or the knock-out of Mstn, the targeted gene by Gasp-2, lead to an overall weight increase of 15% and 28% in mice, respectively (Figure 2B). This gain is associated with an increase in skeletal muscle.

FIGURE 2  Characterization of skeletal muscles from Tg(Gasp-2) mice. A, Total body weight of WT (black, n = 10), Tg(Gasp-2.2) (light grey, n = 10), and Tg(Gasp-2.9) (dark grey, n = 10) were measured from 30 to 90 days old. B, Total body weight of 3-month-old mice (n = 15 mice/genotype). C, Muscles of 3-month-old mice were harvested and weighed. D, Representative cryosections of Tibialis anterior from 3-month-old WT and Tg(Gasp-2) mice. Laminin (red) staining showed basal lamina of myofibers. E, Mean myofiber cross-sectional areas and (F) mean myofiber numbers of Gastrocnemius, Flexor Digitorum Longus (FDL) and Tibialis anterior muscle from 3-month-old WT and Tg(Gasp-2) mice (n = 10 mice/genotype). Data are shown as means ± SEM; One-way ANOVA was performed (WT vs genotypes) (*P value < .05; **P value < .005; ***P value < .001). Benferroni posttest was used for the weight curve to include a correction for repeated measures.
mass of Tg(Gasp-2) lines (gastrocnemius, tibialis anterior, pectoralis, and quadriceps) (Figure 2C). To confirm whether this muscle phenotype is due to hypertrophy and/or hyperplasia, histological analyses were carried out on three muscles (tibialis anterior, soleus, and Gastrocnemius) (Figure 2D-F). Muscle cross sections of Tg(Gasp-2.2) and Tg(Gasp-2.9) mice immunostained with an anti-laminin antibody show an increase of myofiber cross-sectional area (CSA) compared to the WT mice, independently of the muscle type (Figure 2D,E). However, no significant difference in muscle fibers number was observed between WT and Tg(Gasp-2) mice (Figure 2F). This phenotype is found preserved at 6 months (data not shown). These results show that the overexpression of Gasp-2 leads to a myofiber hypertrophy without hyperplasia, as we have previously observed in Tg(Gasp-1) mice.

3.3 Tg(Gasp-2) mice display a switch from slow- to fast-twitch myofibers.

It has been shown that Mstn\textsuperscript{−/−} mice present a switch from slow- to fast-twitch myofibers. We checked whether the Gasp-2 overexpression leads to a change in the myofiber type proportion in two different muscle. We measured the overall activity of isocitrate dehydrogenase ICDH and lactate dehydrogenase LDH from extracts of gastrocnemius and quadriceps of 3-month-old animals (Figure 3A,B). Like Mstn\textsuperscript{−/−} mice, Tg(Gasp-2.2) and Tg(Gasp-2.9) mice show a decrease in ICDH activity compared to WT (Figure 3A). In opposite, Tg(Gasp-1) mice do not present this ICDH decrease but exhibit a decrease in LDH activity (Figure 3A,B). Quantification of the different type of myofibers tends to show a decrease in the percentage of type I myofibers and

**FIGURE 3** Skeletal muscle fiber type distribution from Tg(Gasp-2) mice. A, Isocitrate dehydrogenase (ICDH) and (B) Lactate dehydrogenase (LDH) were measured from Gastrocnemius or quadriceps muscle from 3-month-old WT, Tg(Gasp-2.2), Tg(Gasp-2.9), Tg(Gasp-1), and Mstn\textsuperscript{−/−} mice (n = 10 mice/genotype). The measurement of the LDH and ICDH activities at A\textsubscript{340nm} was based on the NADH disappearance or production, respectively. C, Representative cryosections of Soleus from 3-month-old WT immunostained with an antibody cocktail. D, Percentage of fibers type distribution in the Soleus and Tibialis Anterior, realized by semiautomatic image analysis Visilog software, using the double laminin/myosin labelling (n > 5 mice/genotype). Data are shown as means ± SEM; One-way ANOVA was performed (WT vs genotypes) (*P value < .05; **P value < .005; ***P value < .001)
a significant increase of type IIA myofibers in soleus of Tg(Gasp-2) line (Figure 3C,D). A similar result was observed in Mstn^{–/–} mice but not in Tg(Gasp-1) mice which showed a significant increase in type I fibers and decrease in type IIA myofibers (Figure 3D). We observed the same switch from slow- to fast-twitch in the tibialis anterior muscle (Figure 3D).

### 3.4 | Overexpression of Gasp-2 leads to an increase of myonuclei accretion during the first 3 postnatal weeks

Muscular hypertrophy could be associated to the addition of new nuclei from activated satellite cells within the myofiber and/or to the increased rate of protein synthesis. We therefore analyzed the muscle phenotype of 3-week-old mice, just after the myonuclear accretion phase. The Tg(Gasp-2.2) and Tg(Gasp-2.9) mice already show an increase of muscle mass due to a myofiber hypertrophy without hyperplasia (Figure 4A-C). The number of myonuclei per myofiber was increased in tibialis anterior in Tg(Gasp-2) lines, demonstrating a higher myonuclear accretion (Figure 4D). The pool of Pax7^{+} positive satellite cells are not affected after this myonuclear accretion phase in overexpressing Gasp-2 skeletal muscles (Figure 4E).

### 3.5 | Overexpression of Gasp-2 in Tg(Gasp-2) primary myoblasts enhances cell proliferation and differentiation

To investigate the molecular mechanisms regulating muscle mass in both Tg(Gasp-2) lines which present the same phenotype, myoblasts derived from Tg(Gasp-2.9) satellite cells were isolated. We showed that Tg(Gasp-2.9) myoblasts overexpressed Gasp-2 (a 100-fold change) at 48 hours of proliferation without affecting the Gasp-1 and Mstn expression (Figure 5A). This result is quite surprising since we previously demonstrated that the Tg(Gasp-1) myoblasts showed an upregulation of Mstn. Tg(Gasp-2.9) cells were assessed for rate of proliferation and showed a faster proliferation (Figure 5B), associated with a decrease of pSMAD2/3 (Figure 5C). These results revealed that Gasp-2 overexpression inhibited TGF-β pathway (including Mstn) which normally activated SMAD2/3 phosphorylation.

![Figure 4](https://example.com/figure4.png)

**Figure 4** Characterization of skeletal muscles after myonuclear accretion phase. A, Representative cryosections of Tibialis Anterior (TA) muscle from 3-week-old WT and Tg(Gasp-2.9) mice immunostained for laminin (red), Pax7 (green), and DAPI (blue). B, Mean myofiber cross-sectional areas and (C) mean myofiber numbers of TA muscle from 3-week-old WT and Tg(Gasp-2) mice (n > 5 mice/genotype). D, Quantification of the number of myonuclei per fiber in TA from 3-week-old WT and Tg(Gasp-2) mice and (F) percentage of satellite cells (Pax7^{+}) per cross-sectional area (n > 5 mice/genotype). A nucleus was identified as myonucleus if one of the following criteria is observed: (i) the nucleus was located within the laminin boundary, (ii) the nucleus is at the inner periphery of the fibre (laminin) or (iii) >50% of the surface of the nucleus was within the laminin boundary. Data are shown as means ± SEM; One-way ANOVA was performed (WT vs genotypes) (*P value < .05; **P value < .005)
to inhibit proliferation. Using myosin immunostaining, we observed after 72 hours of differentiation that Tg(Gasp-2.9) myoblasts form larger myotubes compared to WT (Figure 5D). Fusion index of Tg(Gasp-2.9) myotubes is increased, leading to a higher differentiation rate (Figure 5E). In addition, the expression of the two myogenic factors of the terminal differentiation, Mrf-4 and myogenin (Myog), normally inhibited by Mstn, are more expressed in Tg(Gasp-2.9) cells throughout the time course of differentiation (Figure 5F-H).

To obtain more insight into molecular characterization, we performed a gene expression array analysis during proliferation of 43 genes involved in muscle development. Among the 43 genes, 10 genes were upregulated and 5 were downregulated (Table 1). Gasp-2 expression was increased more than 247-fold in the Tg(Gasp-2) myoblasts compared to the WT, confirming that Gasp-2 overexpression was significant in Tg(Gasp-2) satellite cell-derived primary myoblasts. We also found that Myf6, MyoG and Inhba (Inhibin beta A chain) were upregulated in Tg(Gasp-2) cells. In contrast, the Lbhp3 (Latent transforming growth factor beta binding protein 3) gene, another TGF-β inhibitor was downregulated in Tg(Gasp-2) myoblasts (Table 1).
| Gene ID | Gene symbol | Description                                      | Fold changes | P value  |
|---------|-------------|--------------------------------------------------|--------------|----------|
| Upregulated |             |                                                   |              |          |
| 215001 | Gasp-2      | Growth and differentiation factor-associated serum protein-2 | 247.81       | 0.0014   |
| 16323  | Inhba       | Inhibin Beta-A                                   | 2.81         | 0.00364  |
| 16322  | Inha        | Inhibin alpha                                    | 2.74         | 0.00209  |
| 21809  | Tgfb3       | Transforming growth factor B 1                    | 2.48         | 0.0316   |
| 21808  | Tgfb2       | Transforming growth factor B 1                    | 2.33         | 0.0411   |
| 17878  | Myf6        | Myogenic factor 6                                | 2.01         | 0.0134   |
| 17928  | Myog        | Myogenin                                         | 2.01         | 0.00919  |
| 18121  | Nog         | Noggin                                           | 1.98         | 0.0136   |
| 12111  | Bng         | Biglycan                                         | 1.86         | 0.00549  |
| 12667  | Chrd        | Chordin                                          | 1.79         | 0.0573   |
| Downregulated |         |                                                   |              |          |
| 14560  | Gdf10       | Growth and differentiation factor 10             | −1.77        | 0.850    |
| 18505  | Pax3        | Paired box protein 3                             | −1.96        | 0.134    |
| 17927  | Myod1       | Myod1                                            | −2.06        | 0.228    |
| 16998  | Ltbp3       | Latent TGF-b binding protein 3                   | −2.21        | 0.210    |
| 18119  | Nodal       | Nodal                                            | −3.59        | 0.648    |

*Note: List of upregulated or downregulated genes by more than 1.5-fold in Tg(Gasp-2) primary myoblasts compared with WT primary myoblasts during proliferation. One-way ANOVA was performed (WT vs genotypes).*
**TABLE 2** Relative expression levels of deregulated genes in Tg(Gasp-2) mice

| Gene ID  | Gene ID | Gene symbol | Description                                      | Stages          | Embryonic stage—E9.5 | Fetal stage—E14.5 |
|---------|---------|-------------|--------------------------------------------------|------------------|-----------------------|-------------------|
|         |         |             |                                                  |                  | Fold changes | P value | Fold changes | P value |
| Upregulated | 215001 | Gasp-2 | *Growth and differentiation factor-associated serum protein-2* |                  | 22.16       | .02474  | 19.99       | .00717  |
|          | 14561   | GDF-11 | *Growth differentiation factor 11*               |                  | 2.88        | .00874  | 2.22        | .0142   |
|          | 18505   | Pax3    | *Paired box protein 3*                           |                  | 3.07        | .0502   | 3.76        | .0368   |
|          | 18509   | Pax7    | *Paired box protein 7*                           |                  | 2.63        | .0282   | 3.06        | .0254   |
|          | 12159   | Bmp4    | *Bone morphogenetic protein 4*                   |                  | 2.82        | .0263   | 2.38        | .0393   |
| Downregulated | 215001 | Gasp-1 | *Growth and differentiation factor-associated serum protein-1* |                  | −14.12      | .0373   | −2.013      | .0381   |
|          | 14313   | Fst     | *Follistatin*                                    |                  | −2.26       | .0423   | −2.12       | .0454   |
|          | 13179   | Dcn     | *Decorin*                                        |                  | −12.04      | .00482  | −6.11       | .00274  |
|          | 108075  | Ltbp4   | *Latent TGF-b binding protein 4*                 |                  | −3.29       | .0522   | −1.78       | n.s.    |
|          | 12111   | Bgn     | *Biglycan*                                       |                  | −5.22       | .0323   | −2.95       | .0189   |
|          | 16324   | Inhbb   | *Inhibin beta-b*                                 |                  | −5.75       | .0180   | −2.04       | n.s.    |
|          | 16326   | Inhbc   | *Inhibin beta-c*                                 |                  | −8.59       | .00849  | −4.47       | n.s.    |
|          | 12156   | Bmp3    | *Bone morphogenetic protein 3*                   |                  | −6.47       | .0361   | 2.487       | .00647  |

*Note:* Fold change of genes involved in the TGF-β signaling pathway in Tg(Gasp-2) mice are compared with WT mice at embryonic stages E9.5 and E14.5. One-way ANOVA was performed (WT vs genotypes) n.s., non-significant.
Deregulated expression of TGF-β and their inhibitors during primary and secondary myogenesis in Tg(Gasp-2) mice

Understanding the absence of hyperplasia in Gasp-2 overexpressing mice requires to investigate gene expression levels during primary (at E9.5 embryonic stage) and secondary myogenesis (at E14.5 fetal stage). We found a 20-fold overexpression of Gasp-2 at both stages (Table 2 and Figure 6A). Interestingly unlike the Tg(Gasp-1) mice, the Tg(Gasp-2) animals do not present variation in Mstn expression but exhibit at these stages a 2- to 3-fold upregulation of Gdf11, a gene closely related to Mstn known to
regulate anterior/posterior axial patterning (Table 2 and Figure 6B). Moreover, Tg(Gasp-2) mice show a 2- to 14-fold downregulation of several Mstn inhibitors such as Gasp-1, Fst, Dcn, and Ltbp4 at embryonic stages (Table 2 and Figure 6C). Our findings highlighted a gene expression regulatory network of TGF-β members and their inhibitors during primary and secondary myogenesis. This transcriptional deregulation could be responsible for the absence of hyperplasia by counteracting the effect of Gasp-2 overexpression.

3.7 | Overexpressing Gasp-2 mice do not present an adipose and insulin resistance phenotype

We have previously shown that the Tg(Gasp-1) mice gained weight with age due to an increase in fat mass, hyperglycemia, and insulin resistance and found that all these symptoms are dependent of an upregulation of Mstn. At 3 months, the Tg(Gasp-2.2) and Tg(Gasp-2.9) mice do not present changes in adipose tissue mass (subcutaneous, epididymis and brown) compared to controls (Figure 7A). Unlike Tg(Gasp-1) animals, the 16-month-old Tg(Gasp-2) mice show no increase in their fat mass (Figure 7B). In IPGTT, there was no difference in glucose clearance between old mutant and WT mice (Figure 7C). Molecular analyses revealed no upregulation of Mstn in young and aged Tg(Gasp-2) muscles (Figure 7D).

4 | DISCUSSION

In this paper, we studied the cellular and molecular mechanisms underlying the muscle phenotype in a mouse model overexpressing GASP-2, a Mstn inhibitor, to investigate a potential...
new therapeutic approach for muscle atrophy. Although its paralog, GASP-1, was a good candidate because it led to a hypermuscular phenotype when overexpressed, age-related metabolic defects are also observed in Tg(Gasp-1) mice.20 To date, only the phenotypic study of Gasp-2 deficient mice associates in vivo GASP-2 with a context of muscle.26 These knock-out mice develop muscle atrophy and have defects in myofiber regeneration. Here, we generated and characterized two independent lines overexpressing Gasp-2, Tg(Gasp-2.2) and Tg(Gasp-2.9), to better understand the functions of GASP-2 and evaluate its therapeutic potential. We showed that these mice present an increase of skeletal muscle mass due to myofiber hypertrophy at 3 months, still observed at 6 months. This increase is similar to that seen for the Tg(Gasp-1) mice and is less than the observed muscle increase of the Mstn-null mice. We demonstrated that this hypertrophy was accompanied by an increase of myonuclear accretion during the first 3 postnatal weeks. In accordance with these results, we showed that overexpressing Gasp-2 primary myoblasts proliferated faster and myonuclei average per myotube was increased during differentiation. Thus, overexpression of Gasp-2 could result in accelerated regeneration during muscle injury.

Unlike Mstn−/− mice, no muscle hyperplasia was observed in Tg(Gasp-2). We previously observed this absence of hyperplasia in the Tg(Gasp-1) line and have shown an upregulation of Mstn at the embryonic stages, which counterbalances the effect of Gasp-1 overexpression during the early phases of myogenesis.21 Interestingly, we did not find a Mstn upregulation in the Tg(Gasp-2) embryos but an upregulation of Gdf-11, a gene closely related to Mstn known to regulate anterior/posterior axial patterning.27 Recent studies showed that GDF-11 could inhibit skeletal muscle development similar to Mstn.28-30 Differentially upregulation of Mstn or Gdf-11 in Gasp-1 or Gasp-2 overexpressing models could be explained by a different affinity between GASP proteins with Mstn or GDF-11. Indeed, Kondás et al15 and Walker et al31 showed that in vitro, GASP-1 is approximately 100 times more affine for Mstn than GASP-2 and GASP-2 would have a better affinity for GDF-11.15,31 Our in vivo data are consistent with these results and are reinforced by the presence of an up-regulation of Mstn in the Tg(Gasp-1) mice, while Gdf-11 is up-regulated in Tg(Gasp-2) line. In addition, Tg(Gasp-2) mice present a downregulation of several Mstn inhibitors such as Gasp-1, Follistatin, decorin, and Ltbp3 at embryonic stage. A similar result was observed in Tg(Gasp-1) mice, with a downregulation of Gasp-2, Follistatin, and Ltbp1 expression. Our findings highlighted a gene expression regulatory network of TGF-β members and their inhibitors in muscle, responsible for the absence of hyperplasia by counteracting the effect of Gasp-2 overexpression.

Unlike the Tg(Gasp-1) mice,21 GASP-2 overexpression did not lead to metabolic defects with age. In addition, the Tg(Gasp-2) mice display, like the Mstn−/− mice, a switch from slow- to fast-twitch myofibers whereas Tg(Gasp-1) mice exhibit a switch from fast- to slow-twitch myofibers. Altogether, the difference of the phenotypes observed between the Tg(Gasp-1) and Tg(Gasp-2) lines could be explained at the molecular level by the induction or not of Mstn upregulation as shown in Figure 8. Our results suggested that the GASP-2 protein might be a better candidate to target Mstn -signaling pathway without affecting the metabolism. To further develop the potential of GASP-2 as a therapeutic treatment, it would be interesting to get any functional assessment of muscle contractile activity or of muscle regenerative potential.

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DISCLOSURES
The authors have nothing to disclose.

AUTHOR CONTRIBUTIONS
A. Parenté, L. Magnol, and V. Blanquet designed research; A. Parenté, L. Magnol, and V. Blanquet analyzed data; A. Parenté, A. Boukredine, N. Duprat, F. Balaige performed experiments; A. Parenté and V. Blanquet wrote the paper.

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