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Promoter polymorphisms in genes involved in porcine myogenesis influence their transcriptional activity

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Abstract

Background: Success of meat production and selection for improvement of meat quality is among the primary aims in animal production. Meat quality traits are economically important in swine; however, the underlying genetic nature is very complex. Therefore, an improved pork production strongly depends on identifying and studying how genetic variations contribute to modulate gene expression. Promoters are key regions in gene modulation as they harbour several binding motifs to transcription regulatory factors. Therefore, polymorphisms in these regions are likely to deeply affect RNA levels and consequently protein synthesis. In this study, we report the identification of single nucleotide polymorphisms (SNPs) in promoter regions of candidate genes involved in development, cellular differentiation and muscle growth in *Sus scrofa*. We identified SNPs in the promoter regions of genes belonging to the Myogenic Regulatory Factors (*MRF*) gene family (the *Myogenic Differentiation* gene, *MYOD1*) and to Growth and Differentiation Factors (*GDF*) gene family (*Myostatin* gene, *MSTN, GDF8*), in Casertana and Large White breeds. The purpose of this study was to investigate if polymorphisms in the promoters could affect the transcriptional activity of these genes. With this aim, we evaluated *in vitro* the functional activity of the luciferase reporter gene *luc2* activity, driven by two constructs carrying different promoter haplotypes.

Results: We tested the effects of the G302A (U12574) transition on the promoter efficiency in *MYOD1* gene. We ascertained a difference in transcription efficiency for the two variants. A stronger activity of the A-carrying construct is more evident in C2C12. The luciferase expression driven by the *MYOD1*-A allelic variant displayed a 3.8-fold increased transcriptional activity. We investigated the activity of two haplotype variants (AY527152) in the promoter of *GDF8* gene. The *haploptype-1* (A435-A447-A879) up-regulated the expression of the reporter gene by a two-fold increase, and hence presumably of the *GDF8* gene, in both CHO and C2C12 cultured cells.

Conclusions: *In vitro* the *MYOD1*-A allelic variant could up-regulate the expression of *MYOD1* gene. Additionally, we could assess a different response of *in vitro* gene expression according to cell type used to transfect constructs, suggesting that *MyoD* activation is regulated by mechanisms that are specific of myoblasts.

Keywords: *GDF8* gene, *MYOD1* gene, Myogenesis, Promoter, Transcriptional activity, *Sus scrofa*

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Background

Meat quality traits are economically important in swine; however, the underlying genetic control is very complex. For this reason, an improved pork production strongly depends on identifying and studying how genetic variations contribute to modulate gene expression. There is a consistent literature dealing with SNPs affecting coding regions, where the assessment of the possible effect of variation is quite straightforward. However, SNPs within control regions (both at 5′ and 3′) are scarcely studied, although their effect on phenotype might be dramatic. Promoters located upstream genes may extend several bases and initiate transcription. They are key regions in gene expression as they harbour several motifs binding to transcription regulatory factors. Therefore, polymorphisms in these regions are likely to deeply affect RNA amount and consequently protein synthesis. Genes which regulate proliferation and differentiation of precursor cells (myoblasts) into multinucleated myofibers and the consequent formation of muscle tissue (myogenesis), appear likely targets for meat quality determination.

The *MYOD* gene family consists of four structurally related genes: *MYOD1, MYOG, MYF5*, and *MYF6*. The expression of each *MYOD* gene takes place exclusively in skeletal muscles and their products are specific transcription factors which participate in muscle development [1-3]. In particular, products of *MYOD1* and *MYF5* genes are transcription factors that control the processes of myogenesis [3]. Each *MYOD* gene shows a specific pattern during myogenesis: *MYOD1* regulates the embryonic process of mammalian myofiber formation and acts during terminal differentiation of muscle cells activating myogenin [4]. Moreover, phenotypic variations caused by *MYOD1* mutations implicate myofiber-type transformation and myofiber hypertrophy [5,6]. Consequently, polymorphisms within *MYOD1* gene could influence muscle fibers and meat production and quality [1]. Soumillion [7] and then Urbański [8] mapped the porcine *MYOD1* on chromosomes 2. Polymorphisms within porcine MYOD1 have been reported by several groups [3,8,9]. Recently, three SNPs within the porcine *MYOD1* gene in a population of Yorkshire and Berkshire pigs were found significantly associated with meat quality traits, lean meat production and several muscle fiber characteristics such as loin eye area and lightness [10].

The *Myostatin* gene (*MSTN, GDF8)* belongs to the Growth and Differentiation Factors (*GDF*) gene family and negatively regulates skeletal muscle mass development [11]. The *GDF8* gene is expressed during skeletal muscle development both at prenatal and postnatal stages [12,13]. Several studies have verified that mutations in *GDF8* gene cause Double Muscled (DM) phenotype in various cattle breeds [13-16], in sheep [17], in mice [18], in dog [19] and in human [20,21]. Also mutations in cattle *GDF8* promoter affect the muscle conformation [22]. The DM phenotype is characterized by a visible and generalized increase in muscle mass due primarily to hyperplasia [23], an increase in number of cells caused by an excessive proliferation. This muscle condition is opposite to hypertrophy, where cells increase in size and not in number. A polymorphism, located at position 447 (A447G) of the porcine *GDF8* promoter, occurs at a very high rate in the heavily muscled Belgian Piétrain breed. This mutation changes the G nucleotide at position 8 site into an A nucleotide disrupting a putative myocyte enhancer factor 3 binding site [24]. Moreover, it triggers a significantly higher *MSTN* expression in *Longissimus dorsi* of animals that were heterozygous (447AG) for the mutation compared with homozygous wild-type (447AA) and homozygous mutant (447GG) animals [24]. A differential expression of the porcine *GDF8* gene was observed in younger animals. 4 weeks old piglets that were heterozygous (447AG) showed a lower *MSTN* expression than piglets that were homozygous wildtype (447AA). The differentiation of the tertiary myofibres starts at an age of about 4 weeks; at this stage, a lower expression of *GDF8* gene could be correlated with an higher rate of differentiation, resulting in higher muscularity of Piétrain pigs [24].

Gene expression analysis of promoter showed that this mutation can modulate expression levels of the *MSTN* gene in *Longissimus dorsi* skeletal muscle [25]. In commercial lines of pigs, Guimaraes et al. [25] investigated allele frequencies of two SNPs (G435A and A447G), previously identified by Stinckens et al. [26] in the promoter region of the *Myostatin* gene, in complete *Linkage Disequilibrium*. They analysed *MSTN* gene expression pattern in the *Longissimus dorsi* skeletal muscle and performed a statistical association with body composition, carcass composition and meat quality traits. These SNPs were associated to growth and meat quality traits, even though they were not significantly associated with the expression levels of *MSTN* mRNA in muscle. In another study, these two polymorphisms seem to affected growth in Duroc pigs too [27].

In this study, we have chosen known SNPs in the promoter regions of genes involved in development, cellular differentiation and muscle growth, such as the G302A transition [3] in *MYOD1* gene and three polymorphisms, G435A, A447G and T879A, [26] in *GDF8* gene. We investigated if these polymorphisms in the promoter could affect the transcriptional activity of those genes. With this aim, we evaluated *in vitro* the functional activity of reporter gene activity, driven by two constructs carrying different promoter haplotypes, in two different types of cell cultures (epithelial CHO and myoblast C2C12 cultured cells) to check if transcription factors are specific of the type of cell.

Methods

Pig samples and DNA extraction

The Casertana breed is a very ancient Italian autochthonous pig breed reared in Campania, a region of Southern Italy. Large White breed originated in Yorkshire (United Kingdom) and is one of the major pig breeds raised for food all over the world. Casertana and Large White piglets of the same age were reared outdoors in the same environment and fed twice per day with the same commercial diet, following which their productive traits were recorded. Liver tissue was collected at 11 months of age at the time of slaughter. Samples were immediately stored in RNA later until analysis. Animal handling followed the European Union recommendation directive 2010/63/EU and the Italian low 116/92 about animal care. Genomic DNA was extracted from the liver of 19 animals, using Promega Wizard DNA Extraction Kit (Promega Corporation, Madison, Wi, USA) according to manufacturer's instructions, from liver tissue collected at slaughtering and stored at −20°C. DNA was checked for quality on agarose gel and quantified using a DTX microplate reader (Beckman Coulter) after staining with Picogreen (Invitrogen, Carlsbad, CA, USA).

Sequencing analysis and SNP detection

Primer pairs for candidate genes were designed from sequences available at NCBI, using primer3 software http://fokker.wi.mit.edu/primer3 and synthesized by Sigma-Aldrich (Sigma, St. Louis, Mo, USA). The primer sequences, amplicon sizes and positions are reported in Table 1. To sequence PCR fragments and identify new SNPs, the High Fidelity PCR system (Roche, Diagnostic GmbH, Mannheim, Germany) was used following standard conditions by Roche (Buffer 1×, dNTP mix 20 μM, Primer Forward 0.4 μM, Primer Reverse 0.4 μM, 1 μg DNA template, water to volume). A 5 min denaturation step was followed by 34 cycles of denaturation at 94°C (30 sec), annealing for 30 sec and extension at 72°C (1 min); the final extension step was carried out at 72°C for 5 minutes.

PCR products were purified using ExoSap-IT (USB Corporation, Affymetrix) to remove residual primers and dNTPs and used as templates for forward and reverse sequencing reactions. Sequencing was performed using a CEQ8800 sequencer using DTCS QuickStart Kit and purifying with AgencourtCleanSEQ 96 (Beckman Coulter), according to manufacturer instructions. Sequencing of purified PCR products were also outsourced to Macrogen Inc. (www.macrogen.com) for double checking. Sequence analysis and alignments were performed using BLAST; http://www.ncbi.nlm.nih.gov/BLAST) and BioEdit softwares. A total of 19 animals were sequenced, namely, 10 Casertana and 9 Large White pigs.

Cloning of allelic variants into a T/A vector and transformation into competent cells

Fragments harbouring both copies of the target SNPs were cloned using Topo T/A Cloning® in order to separate the two allelic variants (Invitrogen). The T/A cloning and transformation into competent cells (JM109) were performed according to manufacturer's instructions (Invitrogen).

Plasmid constructs were transformed in JM109 competent cells. We mixed 0.5 μl of plasmid in one vial of competent cells. After heat shock step (0°C for 20 min, 42°C for 30 min), we added 250 μl of LB and incubate at 37°C for 1 h. After spinning (3 min at 500 rpm), each vial was put down two LB plate with XGAL, IPTG and Amp and incubate overnight at 37°C. Plasmid minipreparations, for colony screening and transfections, were performed with the PureYield™ Plasmid Miniprep System (Promega).

pGL4.17 constructs

We used the pGL4.17[*luc2*/Neo] vector (Promega), a basic vector with no promoter which encodes the luciferase reporter gene *luc2* (*Photinus pyralis*) as reporter gene and is designed for high expression and reduced anomalous transcription. We inserted the *MYOD1* and *GDF8* amplicons carrying each allelic variants of promoter region, upstream to *luc2* in pGL4.17 vectors. The primer sequences, amplicon sizes of the promoter upstream the coding region are reported in Table 2. The luciferase reporter gene *luc2* (*Photinus pyralis*) was joined to the promoter sequences with matched reading frames. Constructs have been produced by Bio-Fab research (www.biofabresearch.it) and by GeneCust (www.genecust.com).

Table 1 The primer forward and reverse sequences, amplicon sizes and gene positions

Gene	SNP position	Accession number/locus	Forward sequence	Reverse sequence	Amplicon size (bp)	Gene position	Reference
	G302A					promoter	
MYOD1	C489T	U12574	CCCGTCAGTCAGGAGGGACAG	CTTGGGCAGCCGCTGATTCG	612	1st exon	Urbański and Kurył 2004 [3]
	G566C					1st exon	
	G435A					promoter	
GDF8	A447G	AY527152	GCCCTCTGGTCAAATGAGAA	TTTTCCTTTTGCTCGCTGTT	1234	promoter	Stinckens et al. 2005 [26]
	A879T					promoter	

Table 2 The primer forward and reverse sequences, amplicon sizes of promoter upstream the coding region

Gene	SNP position	accession number/locus	forward sequence	reverse sequence	amplicon size (bp)
MYOD1	G302A	U12574	TAGGCTACTACGGG	ATCCCAGCGGGGGCGG	257
GDF8	A447G-A879T	AY527152	GCCCTCTGGTCAAATGAGAA	GATTTTAAAATCAATAC	1139

Cell lines and *in vitro* transfection

A Chinese Hamster Ovary (CHO) cell line and a murine myoblast (C2C12) cell line (both from American Type Culture Collection, Rockville, MD, USA) were used to test the SNP effects in promoter regions. CHO and C2C12 cells were cultured by standard laboratory techniques in Dulbecco's Modified Eagle Medium (Lonza, Lonza Group Ltd, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS) (Cambrex, Bio Science, Walkesville, Md, USA) and 1% l-glutamine (Sigma), only in CHO medium were added 1% MEM, non-essential amino acids (Sigma). Both cell cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C. The day before transfection CHO and C2C12 cells were plated in 24 multi-wells at a density of 9×10^4 and 7×10^4 cells per well, respectively. The day after cells were co-transfected by using Lipofectamine 2000 (Invitrogen), with two different plasmid constructs: the experimental reporter plasmid (pGL4.17[*luc2*/Neo]) and a co-reporter vector, pGL4.74[*hRluc*/TK] vector (Promega) which contains the *hRluc* reporter gene downstream a constitutive HSV-TK immediate-early enhancer/promoter, to normalize the transfection process. The luciferase experimental reporter plasmid (pGL4.17[*luc2*/Neo]) is associated with the effect of each allelic variant (see pGL4.17 constructs), while the activity of the renilla co-transfected reporter vector (pGL4.74[*hRluc*/TK]) provides an internal control to assess transfection efficiency. Briefly, 0.25 μg of plamids (ratio pGL4.17/pGL4.74 up to 20:1) and 1.6 μl of Lipofectamine 2000 were mixed in 100 μl of FBS-free Opti-MEM medium (GIBCO Invitrogen Corporation, Carlsbad, CA, USA) for 20 min and then added to each well containing cells. The cell-plate was then incubated at 37°C for 24 h and each well analyzed for firefly and renilla luciferase activities by bioluminescence analysis.

Bioluminescence analysis

Basically, a reporter assay is a method to translate a biomolecular effect, for example the functional activity of a promoter, into an observable parameter, such as photon production obtained through luminescence. Therefore, the functional activity of promoter variants was analysed *in vitro* with the Dual-Luciferase Reporter Assay System™ (DLR, Promega). In the DLR™ Assay System, the activities of firefly (*Photinus pyralis*) and *Renilla* (*Renilla reniformis*) luciferases were measured sequentially on the same sample, in accordance with the manufacturer's protocol, by using TD-20/20 Luminometer (Promega) and were expressed as relative luminescence units (RLU) per well.

Transfection design experiment

The transfection experimental protocol was designed to assure reproducibility. To this aim, transfection experiments were performed in triplicate and repeated two times by using two mixes (mix A and mix B). Two independent experiments were performed in different days. In order that, each data point is the average of 12 replicates (Additional file 1: Table S1, Additional file 2: Table S2, Additional file 3: Table S3 and Additional file 4: Table S4). The ratio of Firefly to Renilla (FF/R) was taken to represent the normalized firefly luciferase activity. Data are expressed as mean and standard deviation (SD). Negative controls were included.

Statistical analysis

The *in vitro* association between haplotype and transcription efficiency was carried out using y = luciferase/renilla ratio as dependent variable in the model $y = \mu + exp + d + m + r + \varepsilon$, where μ is the overall mean, *d* the day of the experiment (*exp*), *m* the mix (A or B), *r* the replicate (3 for each mix) and ε the residual error. A p value <0.05 (two-tailed) was considered statistically significant. All analyses were performed by R-software version 2.15.1 (http://www.r-project.org/).

Results and discussion

In this study, we have chosen known single nucleotide polymorphisms in the promoter regions of genes involved in development, cellular differentiation and muscle growth; and we investigated if in these SNPs could affect the transcriptional activity of those genes. In order to exclude the complex interactions among genes, we assessed the effects of SNPs in an *in vitro* environment, consisting of only the promoter harbouring one SNP allele and a reporter gene. We analysed the expression levels using an *in vitro* assay, in two different types of cell cultures: a Chinese Hamster Ovary (CHO) cell line and a murine myoblast (C2C12) cell line. The epithelial CHO cells derived from the ovary of the Chinese hamster and are the most commonly used mammalian cultured cells in studies of genetics and medical research. The C2C12 cells are a primary line of murine myoblasts whose behavior corresponds to that of progenitor lineage. That's why the C2C12 cells represent a simplified

muscular tissue and have been used as a model for skeletal muscle development in several works [28].

MYOD1 gene promoter analysis

By sequencing the 5′ flanking region of the *MYOD1* gene, Urbañski and Kury found a transition G302A [3] in the promoter region. In the region that we have analyzed (from 190n of U12574) in our samples, we have found the same polymorphism and no further ones. The functional activity of promoter variants was analysed *in vitro* with the Dual-Luciferase Reporter Assay System. The luciferase reporter gene *luc2* (*Photinus pyralis*) was joined to the promoter sequences with matched reading frames (for details see paragraph *pGL4.17 constructs* in Methods). We monitored the quantitative expression of the reporter luciferase genes (*luc2* and *hRluc*) by luminescence assay (Additional file 1: Table S1 and Additional file 2: Table S2). Figures 1 and 2 show the luminescence results of the G302A variants in CHO cells and C2C12 cells, respectively. The expression of the construct carrying the A allele, is higher in both cell systems, while it is statistically significant only in C2C12. In the latter cells, ANOVA test showed a significant difference between the two variants, *MYOD1-A* and *MYOD1-G* ($p < 0.001$) therefore the A allele up-regulates the expression of *MYOD1* gene in comparison with the G allele. In C2C12 cultured cells, the luciferase expression driven by the *MYOD1*-A allelic variant displayed a 3.8-fold increased transcriptional activity. However, the larger variability in CHO makes the difference between the two variants slightly over ($p = 0.0541$) the threshold we have chosen (0.05).

The stronger activity of the A carrying construct is more evident in C2C12 cells which come from a myoblast line, cultured from the mouse thigh muscle and are competent to differentiate and to express characteristic muscle proteins. Instead, Chinese Hamster Ovary (CHO) cell line derives from ovarian cells of the Chinese hamster. Therefore our data indicate that *MyoD* activation

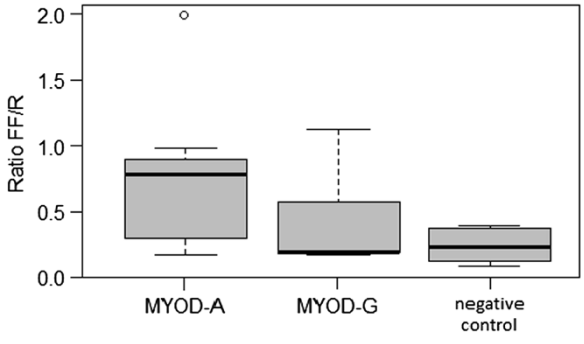

Figure 1 Box plot of the *MYOD1* variant expression in CHO cells. The lines extending vertically from the boxes indicating variability outside the upper and lower quartiles. The outliers are plotted as circles.

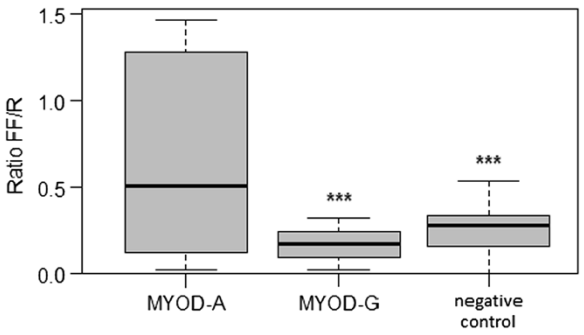

Figure 2 Box plot of the *MYOD1* variant expression in C2C12 cells. The lines extending vertically from the boxes indicating variability outside the upper and lower quartiles.

is regulated by mechanisms that are specific of myoblasts. *MYOD1* shows a muscle-specific gene promoter sequence and transcription is regulated by tissue-specific transcription factors. Extensive studies have identified muscle-specific regulatory factors that drive myogenic differentiation [29]; however, the mechanisms underlying their expression are poorly understood. Several studies have shown the chromatin structure of the chromosomal *MyoD* core enhancer differs between myoblasts and other non-muscle cell types, suggesting epigenetic mechanisms involved in the *MyoD* enhancer repression [30].

GDF8 gene promoter analysis

Previous studies have analysed porcine *myostatin* promoter and identified three polymorphisms: the T879A [31], the G435A and A447G [26]. In our samples we have found the same polymorphisms and no further ones. Additional file 3: Table S3 and Additional file 4: Table S4 show the luminescence results of two haplotype variants (*haplotype-1:* A435-A447-A879; *haplotype-2:* A435-G447-T879) in CHO cells and C2C12 cells, respectively. Figures 3 and 4 show the box plots of the *GDF8* haplotype expression in CHO and C2C12 cells, respectively. The *haplotype-1* up-regulated the expression of the reporter gene by a two-fold increase in both cell systems. The ANOVA test showed that the expression differences between the two variants, *haplotype-1* and *haplotype-2*, are highly significant ($p < 0.001$) in both CHO and C2C12 cultured cells.

These three SNPs were then associated with production traits [24,25,32,33]. Yu et al. [33] reported that these SNPs were associated with early growth traits in Yorkshire pigs; Guimaraes et al. [25] with growth and meat traits in two commercial pig populations (Duroc sires X synthetic white line dams). Stinckens et al. [24] reported a very high allele frequency of A447G in the Pietrain pig breed, for the SNP located at site 447 (A447G); while SNPs G435A and T879A were found to be associated with early growth

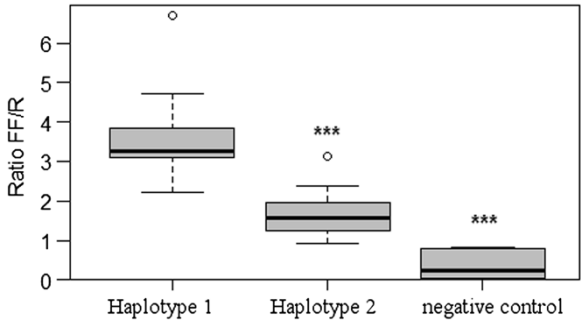

Figure 3 Box plot of the *GDF8* variant expression in CHO cells. The lines extending vertically from the boxes indicating variability outside the upper and lower quartiles. The outliers are plotted as circles.

traits in Duroc pigs and strong linkage disequilibrium exists between them [24]. Liu et al. [34] analysed the associations of this haplotype with birth weight and early growth. A favourable effect on body weight was found for the allele carrying G^{435}-A^{447}-T^{879} [34]. The expression of porcine *myostatin* gene was investigated by qRT-PCR [25,26] and by functional activity of reporter gene [34]. Liu and collaborators found a favourable effect on body weight to work of the allele carrying A^{447}, and that the *myostatin* expression was different between breeds.

In the *GDF8* A447G mutation, the G allele causes the disruption of a putative MEF3 binding site [24]. Several studies described an association of different *GDF8* A447G genotypes with various carcass traits. In Stinckens' paper animals, at an age of 26 weeks, showed a significantly lower *GDF8* expression in homozygous than heterozygous animals [24]; in contrast, in Guimaraes' work, a commercial synthetic line of pigs showed a reduced backfat thickness, a higher lean meat content and less marbling in heterozygous animals when compared with in homozygous wild-type and homozygous mutant animals [25].

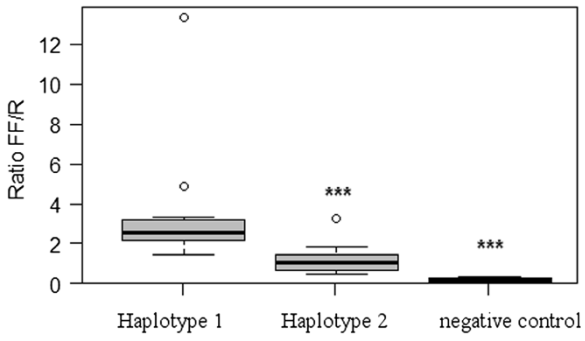

Figure 4 Box plot of the *GDF8* variant expression in C2C12 cells. The lines extending vertically from the boxes indicating variability outside the upper and lower quartiles. The outliers are plotted as circles.

We thus confirm that different haplotypes in the promoter region of *GDF8* gene can modulate its expression. This behavior is shared in both kind of cells. Therefore, we hypothesize that *GDF8* polymorphism interacts with transcription factors that are not muscle specific. Although the major role of myostatin is to suppress myoblast proliferation and myofiber hypertrophy, its function may not be confined to muscle tissue. Jiao et al. [35] detected porcine myostatin mRNA in non muscular tissues such as adipose tissue, heart, liver, spleen, lung kidney and cultured pig fibroblasts, although its expression varied among the different tissues. Myostatin expression has been detected in porcine pituitary gland [23], rat uterus [36], mouse mammary gland [37], adipose tissue [38] and tendons [39]. Myostatin has also been suggested to act as an autocrine factor *in vivo* [40]. Myostatin may play important roles in the development and maintenance of various tissues regulating the energy metabolism and fibrosis [35]. Guimaraes et al. [25] and Stinckens et al. [24] asserted there were only several muscle-related transcription factor binding sites in porcine *MSTN* promoter. Budasz-Swiderska et al. [41] showed that, during terminal differentiation of muscle, the TGF-β1 may control MSTN-related regulation of myogenesis through the up-regulation of MSTN itself. Bing Deng et al. [42] found some adipogenesis- and myogenesis-related transcription factors binding sequence and that MSTN is up-regulated by MyoD and PPARγ, but down-regulated by C/EBPα and C/EBPβ when cells were induced to differentiated into adipocytes.

Conclusion

The goal of this study was to test the effects of single nucleotide polymorphisms (SNPs) in the promoter regions of genes involved in development, cellular differentiation and muscle growth. We sequenced the promoter region of *MYOD1* and *GDF8* genes and we identified the G302A transition in the *MYOD1* gene promoter and two haplotypes in *GDF8* gene promoter. We evaluated *in vitro* the functional activity of reporter gene activity, driven by two constructs carrying different promoter haplotypes, for both genes. In this study, we confirmed that SNPs in the promoter region can modulate *GDF8* gene expression in C2C12 and CHO cells. Particularly, two haplotypes were investigated: *haplotype-1* A435-A447-A879 and *haplotype-2* A435-G447-T879. The *haploptype-1* up-regulated the expression of the reporter gene by a two-fold increase, and hence presumably of the *GDF8* gene, in both CHO and C2C12 cultured cells, suggesting that *GDF8* polymorphisms interact with transcription factors that are not muscle specific.

Furthermore, we could assess that the *MYOD1*-A allelic variant up-regulates the expression of *MYOD1* gene in

C2C12 cells. The stronger activity of the A carrying construct was not statistically significant in CHO cells, suggesting a tissue-specific *MyoD* activation in myoblasts.

Additional files

Additional file 1: Table S1. The *MYOD1* variant expression in CHO cells.
Additional file 2: Table S2. The *MYOD1* variant expression in C2C12 cells.
Additional file 3: Table S3. The *GDF8* variant expression in CHO cells.
Additional file 4: Table S4. The *GDF8* variant expression in C2C12 cells.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
SBo: designed the study, carried out sequencing and cloning experiments, and drafted the manuscript . SBi, MG: carried out sequencing experiments. FT, DW: realized transfection experiments. FI: performed statistical analysis. MSD'A, FP: performed field survey and provided the samples. AV: participated in developing ideas, in supervision and revision of the manuscript. All authors read and approved the final manuscript.

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