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Dr. Xuewen Xu

Unit of Animal Genomics

GIGA-R & Faculty of Veterinary Medicine

University of Liège

Studying the contribution of *PEG11* in determining the callipyge phenotype.

Xuewen Xu, Huijun Chen, Fabien Ectors, Erica Davis, Carole Charlier, Michel Georges & Haruko Takeda.

Unit of Animal Genomics, GIGA-R and Faculty of Veterinary Medicine, University of Liège (B34), 1 Avenue de l'Hôpital, 4000-Liège, Belgium.

Correspondence: michel.georges@ulg.ac.be

We herein describe the generation and characterization of two transgenic lines that ectopically express ovine PEG11 in postnatal skeletal muscle. Line 126 harbors a single copy of the transgene inserted on chromosome 16, while line 127A harbors a complex tandem array of the transgene on chromosome 6. We describe the generation and phenotypic characterization of 213 F2 individuals for line 126. Preliminary analyses indicate that ectopic expression of *PEG11* may indeed cause a muscular hypertrophy (increasing muscle mass by ~5%), and hence participate with DLK1 in causing the muscular phenotype of callipyge sheep.

Introduction

The callipyge phenotype is an inherited muscular hypertrophy of sheep. It is entirely determined by the *CLPG* mutation: an A to G transition affecting a highly conserved dodecamer motif located in the 90Kb intergenic region separating the imprinted *DLK1* and *GTL2* genes on ovine chromosome 18 (Freking et al., 2002; Smit et al., 2003). The *CLPG* mutation inactivates a muscle specific silencer, thereby causing prolonged, ectopic expression of the *DLK1, GTL2, PEG11, PEG11-AS, RIAN*, *MIRG* and associated small RNA genes in skeletal muscle. The *CLPG* mutation does not affect imprinting control of the *DLK1-GTL2* domain: the protein-encoding *DLK1* and *PEG11* remain preferentially expressed from the paternal allele, while the non-coding long *GTL2*, *PEG11-AS*, *RIAN*, and *MIRG*, with embedded small C/D snoRNAs and miRNAs, remain preferentially expressed from the maternal allele (f.i. Charlier et al., 2001; Caiment et al., 2010). As a consequence, the expression pattern of the affected core cluster of genes in the *DLK1-GTL2* domain is distinct in the four genotypes: *+/+*, $+^{Mat}/CLPG^{Pat}$, $CLPG^{Mat}/+^{Pat}$ and *CLPG/CLPG* (Fig. 1).

One of the most remarkable features of the callipyge phenotype is its non-mendelian mode of inheritance known as “polar overdominance”: only $+^{Mat}/CLPG^{Pat}$ animals express the muscular hypertrophy (Cockett et al., 1996). The distinctive feature of $+^{Mat}/CLPG^{Pat}$ animals is ectopic expression of the protein coding *DLK1* and *PEG11* genes in the absence of expression of the long and small non-coding RNA genes. DLK1 encodes a member of the Notch-Delta family of membrane receptors/ligands, which is known to be involved in adipogenesis, haematopoiesis, neurogenesis and adaptation to independent life

(f.i. Georges et al., 2013). We induced ectopic expression of DLK1 in skeletal muscle of transgenic mice, and this resulted in a muscular hypertrophy. Hence, we concluded that ectopic expression of DLK1 is the cause of the callipyge phenotype (Davis et al., 2004). However, it has been suggested by some (f.i. Byrne et al., 2010; Anne Fergusson-Smith, personal communication) that PEG11 might also be involved in the determinism of the callipyge muscular hypertrophy. PEG11 is an "exapted" protein derived from a sushi-ishi retrotransposon, which is essential for placental development (Sekita et al., 2008). Of note, PEG11 was recently shown to be a driver in hepatocarcinogenesis (Riordan et al., 2013). The aim of this work was to address this question and to study the contribution of ectopic PEG11 expression to the determinism of the callipyge muscular hypertrophy.

The absence of phenotypic expression in *CLPG/CLPG* animals is thought to reflect post-transcriptional inhibition of the *DLK1* (and possibly *PEG11*) transcripts by non-coding RNA genes expressed from the maternal allele (Fig. 1). This would account for the low levels of DLK1 protein in skeletal muscle of *CLPG/CLPG* when compared to $+^{Mat}/CLPG^{Pat}$ individuals (Davis et al., 2004; White et al., 2008). Intriguingly, *PEG11* trans-inhibition was indeed shown to occur and to be mediated by miRNAs processed from the *PEG11-AS* pri-miRNA (Davis et al., 2005). The mediators of the trans-inhibition of *DLK1* remain unknown (reviewed in Georges et al., 2013).

Results

Generating mice harboring an ovine (o)PEG11 transgene. To study the effect of ectopic PEG11 expression in skeletal muscle, we generated transgenic mice following Davis et al. (2004). The 1,333 amino acid long ovine *PEG11* open reading frame (ORF) was cloned in a modified pMLC3F-nlacz-2E vector (Davis et al., 2004), placed under the dependence of the murine myosin light chain 3F promoter and 2E enhancer. The corresponding control elements are expected to drive expression of the transgene in type IIB muscle fibers throughout pre- and postnatal development. The 7.5Kb insert was released from the remainder of the vector by digestion with *Not*I and *Sma*I, gel-purified and microinjected into fertilized FVB eggs, which were re-implanted into pseudopregnant (C57BL6J× CBA) F1 foster mothers. We obtained 41 offspring, of which eight proved to carry the transgene upon PCR analysis of tail DNA. When mated to FVB mice, six of the eight founders transmitted the transgene to offspring. We performed RT-PCR targeting the ovine PEG11 ORF in *Quadriceps femoris* of two offspring per transmitting founder. Transgene expression was observed for two lines, referred to as 126 and 127, and these were maintained for further analysis.

Characterizing integration site, segregation and expression of the oPEG11 transgene in lines 126 and 127A. We combined Southern blotting, regular PCR, and "splinkerette" PCR to characterize the transgene insertions in lines 126 and 127.

Line 126 was shown to harbor one single-copy transgene integrated in an intergenic region on chromosome 16, at ~407Kb from *Robo2* (*Roundabout homolog 2*) and ~722Kb from *Lipi* (*lipase, member I*). Unexpectedly, the

transgene comprised both the *MLC/oPEG11* insert, as well as the remainder of the vector including a 0.22 Kb duplicated segment flanking the transgene on either end (Fig. 2). This indicated that the microinjected insert preparation was contaminated with undigested vector. The restriction patterns obtained by Southern blotting suggest that the transgene insertion was accompanied by a ~ 1.9 Kb deletion at the integration site.

Line 127 was shown to harbor two distinct integration sites (127A and 127B), both comprising a very similar, complex array thought to encompass multiple tandem copies of digested insert and undigested full-length vector, confirming the contamination of the insert preparation with undigested vector. The 127A integration site was shown to correspond to the last intron of the *Ccdc91* (coiled-coil domain containing 91) gene on chromosome 6 (Fig. 3). Expression of the transgene was only observed in mice with the 127A integration, not in mice with the 127B integration.

We developed PCR-based genotyping assays for the 126 and 127A transgenes using the sequences of the identified integration sites (Fig. 2&3), and generated both back- (T+ x ++) and intercross (T+ x T+) populations for a total of 487 (line 126) and 87 (line 127A) offspring (Table 1). The transgene tended to be over-transmitted in the line 126 back-cross ($p = 0.02$), while under-transmitted in the line 127A back- ($p = 0.02$) and inter-cross ($p = 0.05$). The absence of homozygous TT offspring in the 127A inter-cross suggests that they succumb during pregnancy, which could result from *Ccdc91* knock-out. The causes of the over- (line 126) and under-transmission (line 127A) in the back-cross matings remain unknown.

We used RT-PCR, RACE and Northern blotting to study the expression of the transgenes at the RNA level (Fig. 4). RT-PCR analysis indicated that the transgene was preferentially expressed in skeletal muscle, and that expression increased from birth to 10 weeks in this tissue. In addition, detectable levels of expression were observed in heart, lung, liver, kidney and spleen. Northern blotting using total RNA extracted from *Quadriceps femoris* of 10-week old animals revealed transgene-specific bands of expected size (~4.3Kb), albeit apparently slightly larger in line 127A when compared to line 126. RT-PCR analysis with overlapping amplicons revealed amplification products of expected size and sequence for line 126. It is noteworthy that the PCR yield for the most 3' amplicon was lower than expected; the significance of this finding is being examined. Similar results were obtained for line 127A with, however, extra amplification products for the 5' amplicon. The largest of these was shown by sequencing to result from retention of the intron. 5' and 3' RACE uncovered the expected, vector-dependent transcription start and polyadenylation sites for both transgenes.

Studying the phenotypic effects of the oPEG11 transgenes in lines 126. Given the fact that line 126 expressed an apparently functional oPEG11 transcript in skeletal muscle we focused our initial phenotypic analysis on the corresponding F2 population (Table 1). The 213 animals were euthanized at 70 days and dissected. We measured body weight as well as the weight of the right and left quadriceps femoris, right and left triceps brachialis, right and left kidney, spleen, liver, and heart. We analyzed the effect of the transgene on these phenotypes separately for each sex, using a mixed model that included the fixed effect of

transgene genotype (++, T+ or TT) and a random litter effect (Fig. 5). We observed a significant positive effect of the oPEG11 transgene on the weight of the quadriceps (p=0.0004) and triceps (p=0.01) in females, and a suggestive positive effect on the weight of quadriceps (p=0.054) in males. The weight of the quadriceps was increased by ~5% in TT females when compared to controls. There was a non-significant trend towards increased triceps weight in transgenic males. The effects on the weight of other organs were not significant, with the exception of an overdominant effect on lung weight in males (p=0.02; data not shown). Taken together, these preliminary results suggest that ectopic expression of PEG11 in skeletal muscle may indeed cause a muscular hypertrophy, and hence possibly contribute to the callipyge muscular hypertrophy. It is noteworthy that the effect of the oPEG11 transgene appeared to be recessive in females, yet dominant in males (Fig. 5).

Discussion

We herein describe the generation and characterization of two lines of transgenic mice that ectopically express ovine PEG11 in skeletal muscle, hence mimicking one specific feature of the transcript profile observed in the $+^{Mat}/CLPG^{Pat}$ callipyge sheep. Our results suggest that ectopic expression of PEG11 may contribute to the callipyge muscular hypertrophy in addition to the previously demonstrated effect of DLK1 (Davis et al., 2004).

A number of experiments are underway to consolidate these initial findings. More specifically, we are: (i) increasing the size of the 126 F2 cohort, (ii) analyzing F2 cohort for line 127A, (iii) performing histological analysis of

skeletal muscle to determine whether the observed, macroscopic hypertrophy results from an increase in the number and/or diameter of the muscle fibers, and (iv) analyzing the expression of the oPEG11 transgene at the protein level.

In addition, we are generating a cohort that simultaneously segregates for the DLK1 and oPEG11 transgenes, which will allow us to test for a possibly synergistic effect between these two genes.

The mechanism by which DLK1 and PEG11 might cause the observed muscular hypertrophy remains unknown. We cannot exclude that ectopic expression of proteins that are not normally expressed in skeletal muscle trigger a non-specific response which is accompanied by an increase in muscle mass. Transcriptome analyses could be conducted to study the downstream effects associated with ectopic expression of the corresponding transgenes.

Methods

Transgene construction. The complete ovine PEG11 ORF was amplified by PCR from as seven partially overlapping fragments from BAC 229G11 (Segers et al., 2000; Charlier et al., 2001a; Smit et al. 2005). These were digested with restriction enzymes (*BspeI, BglI, NsiI, AatII, BspHI, MluI*) in order to generate paired cohesive ends which allowed for the reconstitution by ligation of a 4,178 bp fragment spanning from codon two to 175 bp downstream of the TGA stop codon of the ovine *PEG11* gene. The corresponding fragment was digested with *BglII* and ligated in frame with the vector's ATG initiation codon in the pMLC3F-nlacZ-2E vector (Davis et al., 2004). A 7.5 Kb fragment encompassing MLC 3F promotor, ovine PEG11 ORF and MLC enhancer was released from the remainder

of the vector by digestion with *Not*I and *Sma*I, subjected to agarose gel electrophoresis, and purified with the Gene Clean Spin procedure (Polylab).

Generation of transgenic mice. Transgenic mice were generated following Davis et al., 2004. Briefly, transgenic mice were generated by pronuclear microinjection of purified DNA fragments into fertilized FVB eggs at the concentration of 1ng/μl via standard techniques. Injected eggs were then reimplanted into pseudopregnant(C57BL/6J X CBA) F1 foster mothers. The transgene-positive founders were mated with wild type FVB mice to produce F1 offspring used for further analyses.

Characterizing the transgene insertion sites. (i) Southern blotting: Genomic DNA was extracted from Proteinase K digested mouse spleen using standard phenol/chloroform extraction followed by ethanol precipitation. Ten micrograms of genomic DNA were digested overnight at 37° C with *Spe*I and *Mfe*I , and separated on a 0.8% agarose gel. The gel was equilibrated in the transfer buffer (0.4N NaOH and 1M NaCl) and DNA was capillary transferred to Hybond N+ membranes (GE Healthcare). Probes were obtained by PCR amplification from the vector, labeled with ^{32}P-dCTP with the Prime-It II random primer labeling kit (Agilent), and hybridized with the membrane in ULTRAhyb ultrasensitive hybridization buffer (Ambion) at 55° C overnight. After washing, the membrane was exposed to a phosphor screen which was subsequently scanned on a Typhoon 9400 instrument (GE Healthcare). (ii) PCR: PCR amplifications were conducted using the primers reported in Suppl. Table1, and Phusion Hot Start II High-Fidelity DNA Polymerase (Finnzymes) under the following thermocyling conditions: (1) 98 ° C 2min, (2) 5 cycles of 98 ° C for 10s,

62 °C for 20s and 72 °C for 50s, (3) 5 cycles of 98 °C for 10s, 60 °C for 10s and 72 °C for 50s, (4) 25 cycles of 98 °C for 10s, 58 °C for 10s and 72 °C for 40s, followed by (5) a 5 min final extension at 72 °C. The PCR products were analyzed by electrophoresis in a 1.5% agarose gel. (iii) Splinkerette PCR: Splinkerette PCR was conducted following Potter & Luo (2010). Briefly, 1µg of genomic DNA was digested with *Fat*I (line 126) or ApoI (line 127) and ligated to corresponding complementary linkers (Suppl. Table 1). Two rounds of nested PCR were conducted using primers reported in Suppl. Table 1, and gel-purified amplification products sequenced using standard procedures.

Developing genotype assays for lines 126 and 127A. Based on the knowledge of the 126 and 127A transgene integration sites, we designed allele-specific PCR assays for both lines using the primer pairs reported in Suppl. Table 1. PCRs were performed using Go-Taq DNA polymerase (Promega) under the following cycling condition: (1) 95 °C 3min, (2) 35 cycles of 95 °C for 30s, 60 °C for 30s and 72 °C for 30s, followed by (3) a final extension at 72 °C for 5min. Amplification products were pooled and loaded on 2.5% agarose gels for genotyping.

Characterizing the expression of transgenes 126 and 127A at the RNA level. (i) RNA extraction: Samples for expression analysis included quadriceps femoris of two days, two weeks and 10 weeks old males, as well as heart, lung, spleen, liver and kidney from 10 weeks old males. Total RNA was extracted using Trizol^{R} according to the manufacturer's instructions (Invitrogen). RNA was treated with Turbo^{TM} DNase (Ambion) prior to RT-PCR or RACE. (ii) Northern blotting: Twenty five µg of total RNA was denatured and electrophoresed using 1%

agarose gels containing 2M formaldehyde. The gel was soaked for 15 min in 50 mM NaOH and transferred to Hybond N+ membranes (GE Healthcare) with 10× SSC and RNA was UV-cross linked with the membrane after transferring. A 1,026bp ovine PEG11-specific probe was generated by PCR (Suppl. Table 1) and labeled with ^{32}P-dCTP. After an 16 hour hybridization at 45° C in ULTRA ultrasensitive hybridization buffer(Ambion), the membranes were washed and exposed to a phosphor screen for one week then scanned on Typhoon 9400 (GE Healthcare). (iii) RT-PCR: cDNA synthesis was conducted using 1 μg of total RNA primed with 40 pmol oligo dT_{18}, 40 pmol random primers and 1 pmol of each two gene-specific primers (Suppl. Table 1) and RevertAid™ H Minus reverse transcriptase (Fermentas). For regular PCR, we used three distinct primer pairs (Suppl. Table 1) to amplify three partially overlapping amplicons spanning oPEG11 using Phusion Hot Start II High-Fidelity DNA Polymerase (Finnzymes) under the following thermocyling conditions: (1) 98 ° C 1min, (2) 5 cycles of 98 ° C for 10s, 65 ° C for 20s and 72 ° C for 90s, (3) 5 cycles of 98 ° C for 10s, 60 ° C for 10s and 72 ° C for 80s, (4) 25 cycles of 98 ° C for 10s, 58 ° C for 10s and 72 ° C for 70s, followed by (5) 5 min final extension at 72 ° C. The PCR products were purified after separation on 1.5% agarose gels and validated by sequencing. For qRT-PCR, the cDNA was prepared as described above, but primed by 50 pmol oligo dT_{18} and 50 pmol random primers from 1μg of total RNA. The qRT-PCR assay was conducted for both ovine PEG11 and HPRT as internal control on a 7900HT Sequence Detection System (ABI) under the following conditions: (1) initial denaturing at 95° C for 15 min, followed by (2) 40 cycles of 95° C 15 s, 60° C 30 s, 72° C 30 s, and (3) a melting curve analysis with 95° C 15 s, 60° C 15 s, 95°

C 15 s. (iv) RACE: cDNA for 5' and 3'RACE was prepared with the GeneRacer Kit (invitrogen) from 5 μg and 1μg total RNA respectively. 5'RACE was performed using Phusion Hot Start II High-Fidelity DNA Polymerase (Finnzymes) using an oPEG11 specific primer (Suppl. Table 1) and the GeneRACE®5'primer, under the following conditions: (1) 98° C 30 s, (2) 4 cycles of 98° C 10 s, 70° C 30 s, (3) 4 cycles of 98° C 10 s, 68° C 30 s, (4) 25 cycles of 98° C 10 s, 65° C 10 s, 72° C 30 s and (5) a final extension at 72° C for 5 min. 3'RACE was conducted with a gene-specific primer (Suppl. Table 1) paired with GeneRACE®3'primer under the same condition as 5'RACE. The RACE products were gel-purified and validated by sequencing.

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1 **Table 1:** Transgene segregation (back- and inter-cross) in lines 126 and 127A

Parents		Offspring			
		++	T+	TT	p[#]
126	T+ x ++	118	156	-	0.02
	T+ x T+	56	108	49	0.78
127A	T+ x ++	45	26	-	0.02
	T+ x T+	4	12	-	0.05

2 [#] p-value assuming Mendelian segregation of the transgene.

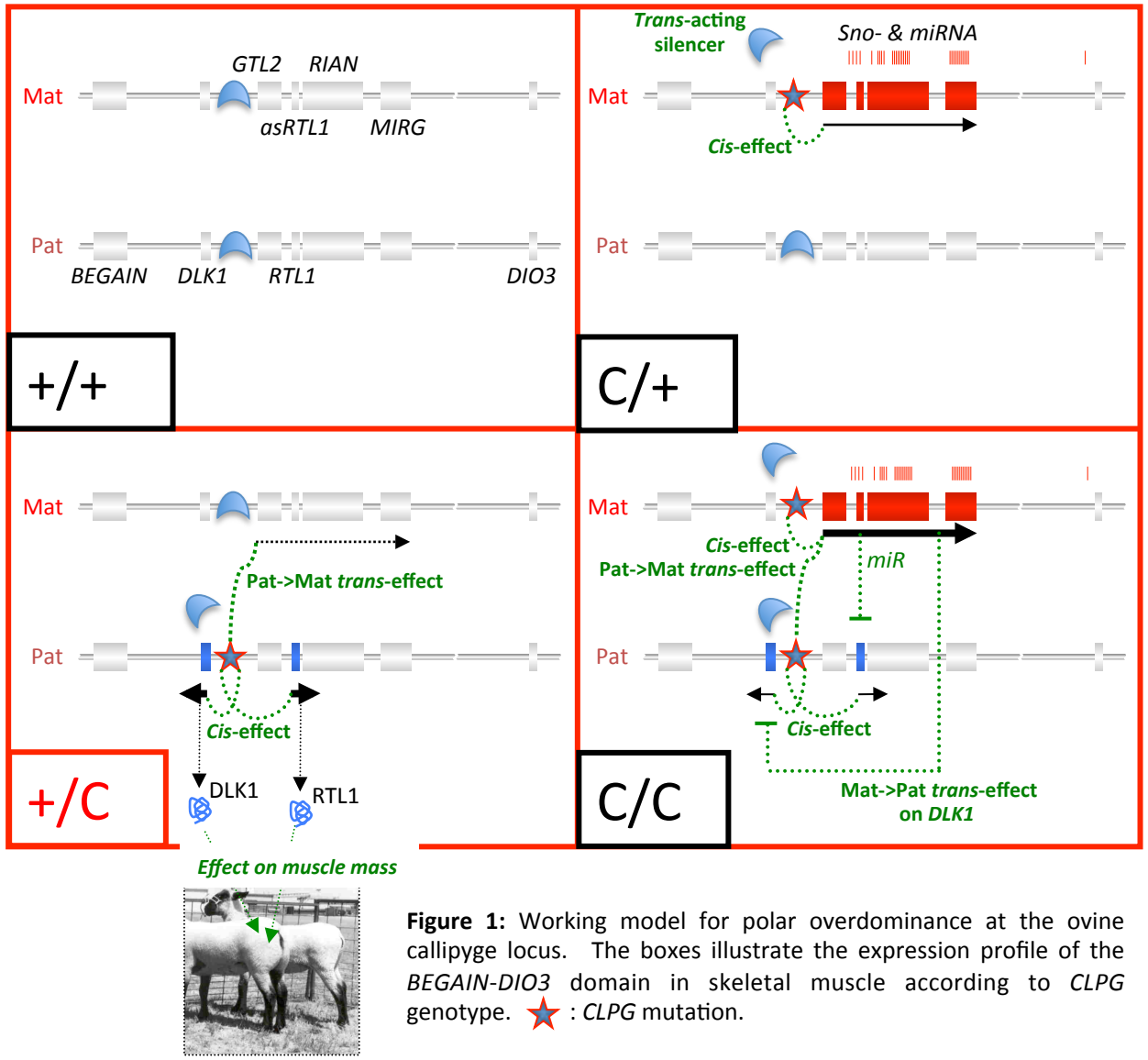

Figure 1: Working model for polar overdominance at the ovine callipyge locus. The boxes illustrate the expression profile of the *BEGAIN-DIO3* domain in skeletal muscle according to *CLPG* genotype. ★ : *CLPG* mutation.

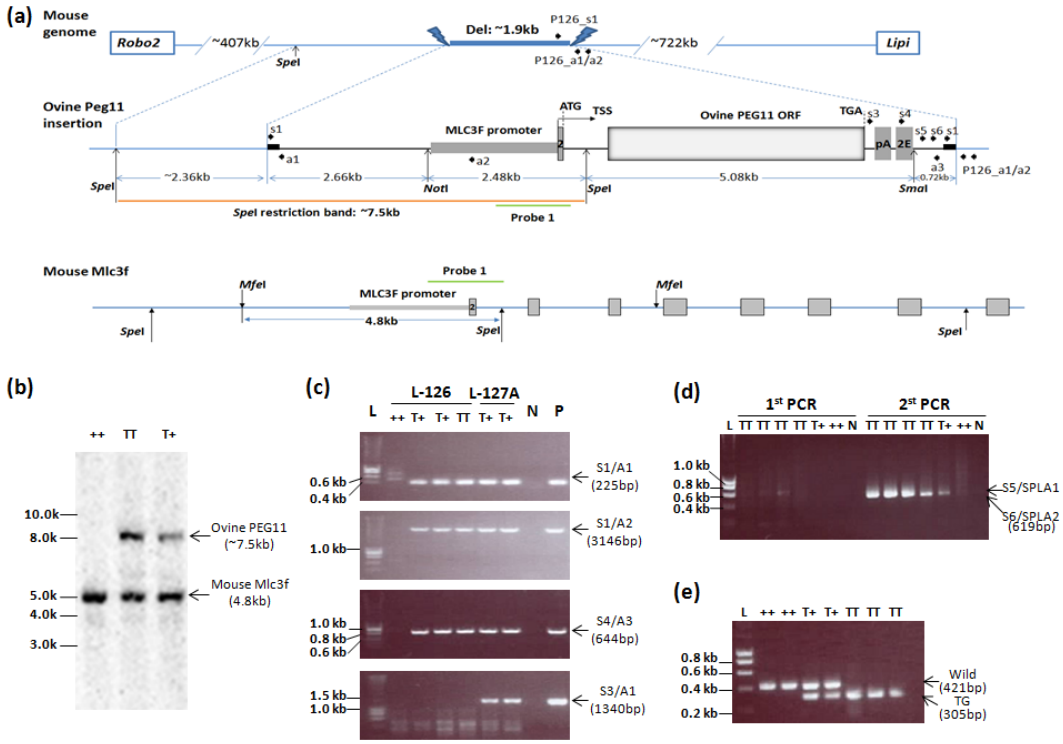

Figure 2. Characterization of the transgene integration site in line 126. (a) *Spe*I-*Mfe*I restriction map of the transgene in line 126, as well as of the endogenous mouse MLC3F gene. The mouse genome is represented by a blue line, and the transgene insertion is shown in black and grey. MLC components include "3F promoter" (thin grey box), exon 2, intron2 and 2E enhancer. The ovine PEG11 ORF is represented by a long grey box. "TSS" represents the expected transcription ignition site, "pA" is the bovine growth hormone polyadenylation site. "ATG" and "TGA" indicate the "start" codon and "stop" codon of the transgene. The two small black boxes at the two end of the insertion represent a 220 bp duplication flanking the whole construct. The transgene insertion is supposed to be accompanied by a ~1.9kb deletion represented by a thin blue box. The probe used for Southern blotting is labeled as a blue horizontal bar, and the expected target fragment is represented by a yellow line. The position of the primers for regular PCR and "splinkerette" PCR are labeled with arrows. (b) Southern blotting result of line 126. The fragment size of the endogenous MLC3F is 4.8kb, and the transgene specific fragment is approximately 7.5kb. For heterozygotes, the average ratio of band intensities for the transgene and endogenous control is ~0.52, indicating a single-copy transgene insertion. (c) Regular PCR amplification revealed the existence of undigested plasmid at both ends. "L" is the smart ladder, "N" is the negative PCR amplification control (water), "P" represents the positive control with transgene construct plasmid DNA. (d) "Splinkerette" PCR amplification results. The PCR product of the second round of amplification was sequenced. (e) PCR genotyping results, the bigger band (421bp) corresponds to the wildtype allele and the smaller band (305bp) corresponds to the transgenic-bearing allele.

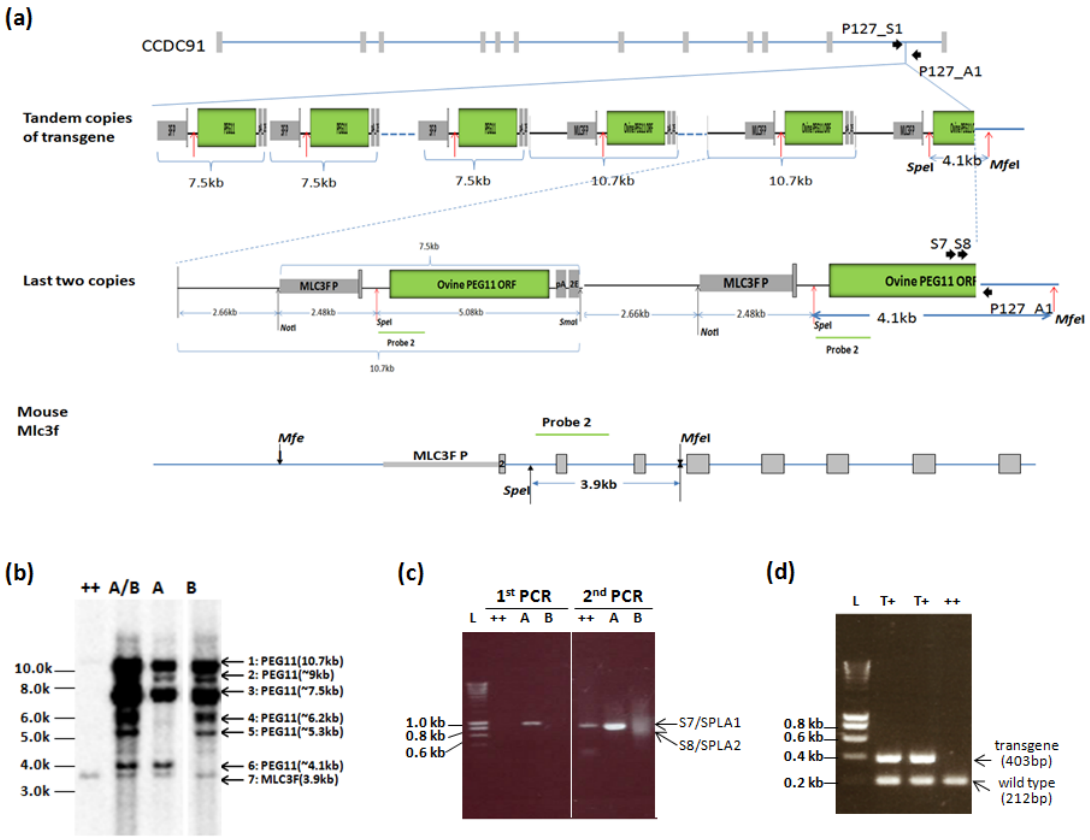

Figure 3. Characterization of the transgene integration for line 127A. (a) Hypothesized structure and *Spe*I-*Mfe*I restriction map of the integration site, as well as *Spe*I-*Mfe*I restriction map of the endogenous mouse MLC3F gene. The hybridization sites of the probe used in the Southern blotting are labeled as green horizontal bars. "S7" and "S8" are primers used for "splinkerette PCR", and "P127_S1", "P127_A1" are primers used for genotyping. (b) Southern blotting result for line 127A. Samples were digested with both *Spe*I and *Mfe*I. The fragment size of the endogenous mouse MLC3F is 3.9kb. For line 127A and Line 127B, there are four common bands, two dark transgene specific fragments, one lighter band (fragment 2: ~9kb) in the middle of two major bands and the band of endogenous MLC3F (fragment 7: 3.9kb). The smaller dark band (fragment 3) is approximately 7.5kb, possibly corresponding to the linear fragment produced by *Not*I and *Sma*I digestion, the bigger one (fragment 1) is approximately 10.7kb, possibly corresponding to the full-length construct. The lighter band (fragment 2) in the middle of two dark bands is approximately 9kb, possibly corresponding to the linker of the digested linear fragment (7.5kb) and the full-length copy (10.7kb). For line 127A, one specific band is approximately 4.1kb (fragment 6), and for line 127B, there are two specific bands (fragment 4 and 5). (c) "Splinkerette" PCR amplification results of line 127A. (d) PCR genotyping result of line 127A, the transgene allele (403bp) was amplified by S8/P127_A1, and the wildtype allele (212bp) was amplified by P127_S1/A1.

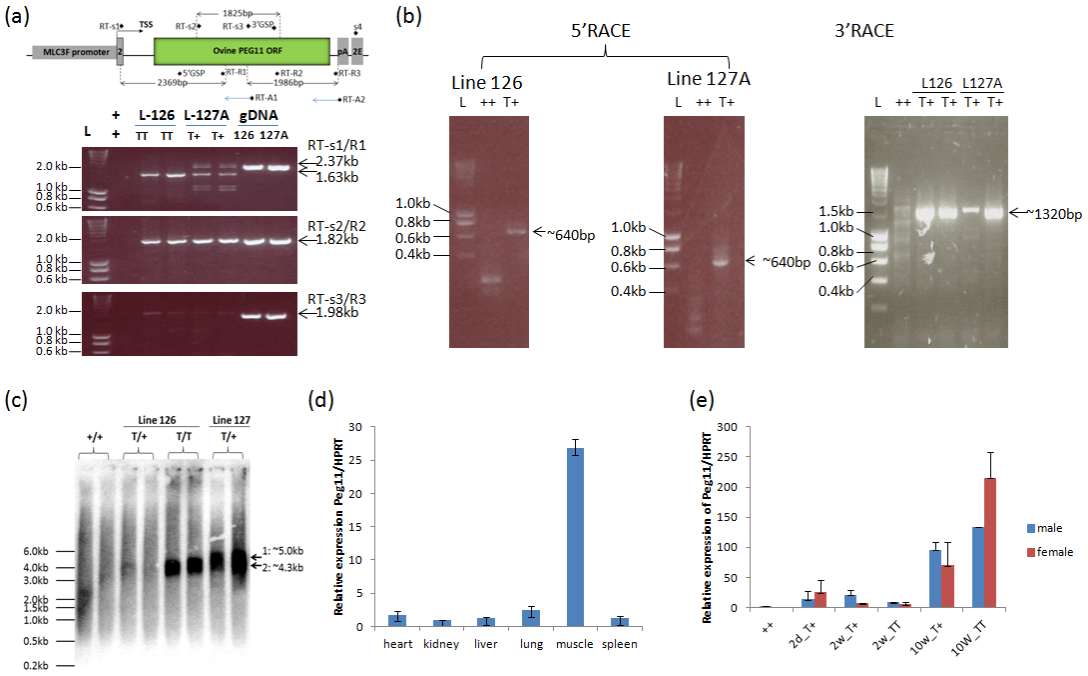

Figure 3. Expression analysis. (a) RT-PCR. The location of three pairs of primers were shown in the previous diagrams. "L" is the smart ladder LW-1700(Eurogentec). The first five samples are skeletal muscle cDNA from one wildtype FVB mice (++), two transgenic homozygotes of line 126 (TT) and two transgenic heterozygous of line 127 (T+). "gDNA" represent genomic DNA. (b) 5'RACE and 3'RACE. (c) Northern blotting. One specific band (approximately 4.3kb) was detected in the transgenic homozygotes of line 126, and a relatively bigger band (approximately 5kb) was detected in line 127. (d) qRT-PCR detection of the ovine PEG11 expression in different tissues (n=3). (e) Temporal expression pattern of the ovine PEG11 in quadriceps muscle of line 126. "+/+": wildtype FVB mice, "2d_T/+": transgenic heterozygote mice of 2 days of age, "2w_T/+": transgenic heterozygote mice of 2 weeks of age. The blue bars correspond to males and the red bars to females.

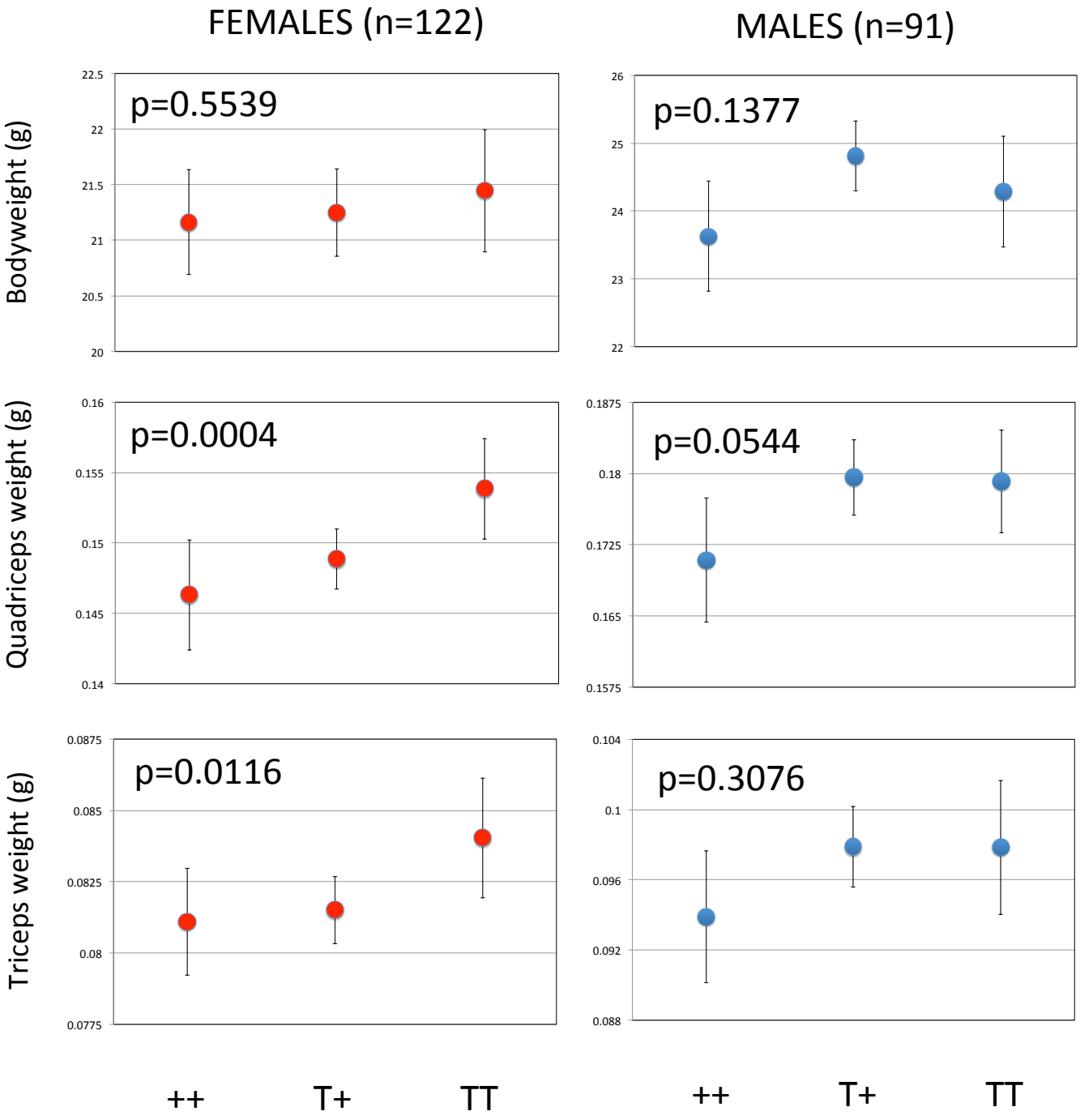

Figure 5: Effect of the oPEG11 transgene on body weight, weight of both quadriceps, and weight of both triceps in line 126.

Supplemental Table 1: Utilized Primers

Primer name	Primers sequence(5'-3')	Application
S1	ggaacaacactcaaccctatctc	normal PCR
A1	cggcatcagagcagattgtac	
A2	agccctaaatctgaattgccta	
S3	ctgagaaagggcacgcaaccaact	
S4	gcaggcagaagagtggtagtca	
A3	aaaccgtctatcagggcgatg	
P126_S1	ggtgactcccataacatccgt	Genotyping
P126_A1	gaaggaagaggttccgtctgt	
P126_A2	tgctctctaatggcgtgtgcta	
P127_S1	acacgctggtcttggacgga	
P127_A1	agcaacctggaaatgacagcag	
Peg11-probe1-S1	cccatgtatgaactccagtgaac	Probe
peg11-probe1-A1	gtcctccatcctattccttgtctg	amplification
Peg11-probe2-s1	gtataccttatctgacatatgcta	
Peg11-probe2-A1	gtcatcaatcatggagctttc	
Peg11-norp-S	ttgagacgatgatggagcgtaaga	
Peg12-norp-A	tctcagccagcttgtcctctatct	
S5	ccctcctttctcgccacgttc	Splinkerette
S6	ctttagggttccgatttagtgct	PCR
S7	cttcttcgcccacttcaactttgac	
S8	ccaccaccaccaggccaacgatg	
SPLK-A	cgaagagtaaccgttgctaggagagaccgtggctgaatgagactggtgtcgacactagtgg	
SPLK-B	**catg**ccactagtgtcgacaccagtctctaatttttttttttcaaaaaaa	
SPLK-E	**aatt**ccactagtgtcgacaccagtctctaatttttttttttcaaaaaaa	
SPLA1	cgaagagtaaccgttgctaggagagacc	
SPLA2	gtggctgaatgagactggtgtcgac	
RT-A1	tagcggaagcggaccagaac	RT-PCR
RT-A2	acaacagatggctggcaactagaa	
RT-S1	cgcagcctagaactccatcatg	
RT-R1	cagtaggcttacggcatggtgtt	
RT-S2	gctcaaggaagacatcaggcagta	
RT-R2	gtagcggcaccacaccatgaa	
RT-S3	tgagccaggaggagcattacca	
RT-R3	actgttgctggttgatgagttggt	
qRT-S	ggcagaggctccgcaaga	qRT-PCR
qRT-A	cgttttctgattctggggactg	
HPRT-S	agctactgtaatgatcagtcaacg	
HPRT-R	agaggtccttttcaccagca	
5GSP	gctggcagagcacgatgaact	RACE
3GSP	gacgtcaaggagatgccgaca	