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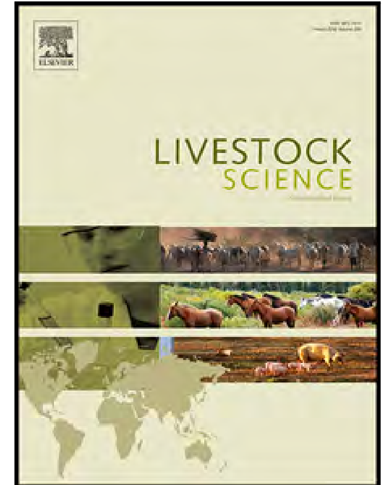

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Highlights

- Alfalfa lambs showed lower levels of *SCD* gene expression than indoors lambs.
- The *SCD* gene expression was affected by the rs412429481 SNP.
- The *SCD* promoter with the A allele had higher activity by luciferase and gene expression assays.

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A functional variant in the *stearoyl-CoA desaturase* (*SCD*) gene promoter affects gene expression in ovine muscle

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Abstract

The nutritional quality of lambs may be improved with increased *stearoyl-CoA desaturase* (*SCD)* gene expression, which increases the desaturation of stearic acid to oleic acid. The aim of this study was to evaluate the effect of the rs412429481 (FJ513370: g.31C>A) SNP located at the *SCD* gene on the functionality of the gene in lambs reared under different production systems. The effect of the rs412429481 SNP on gene expression in Rasa Aragonesa male lambs slaughtered at 22-24 kg was studied in two experiments. In Experiment 1 (n=44), the *semitendinosus* muscle of lambs grazing alfalfa (ALF) or fed concentrates indoors (IND) was analysed; in Experiment 2 (n=48), the *semitendinosus* and *longissimus thoracis* muscles of lambs that received supplementation with dl-α-tocopheryl acetate for different finishing periods were used. In Experiment 1, the effect of the rs412429481 SNP on the expression of the *SCD* gene in the *semitendinosus* muscle depended on the feeding group (P<0.001), as it had no effect in ALF lambs, but CA lambs had greater *SCD* expression than CC lambs under the IND conditions. Moreover, ALF lambs showed lower levels of *SCD* gene expression than IND lambs (*P*<0.05). In Experiment 2, gene expression was affected by the rs412429481 SNP in both muscles. Animals carrying the C- allele showed a lower expression rate than animals carrying the A- allele. These different expression levels were not associated with changes in the DNA methylation pattern or by the binding of specific nuclear proteins. Finally, we confirmed these results by luciferase assays, demonstrating that the *SCD* promoter containing the A variant had a 23.9% higher activity than the promoter containing the C variant.

Keywords: sheep; SCD; nutrigenetics; functional; fatty acid profile.

1. Introduction

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The conjugated linoleic acid *cis* 9-*trans* 11 isomer (CLA) has been associated with numerous health benefits for consumers, including the prevention of atherosclerosis, hypertension and even different types of cancer (Bhattacharya et al., 2006). In the same context, an increased ratio of mono-unsaturated fatty acids (MUFAs) to saturated fatty acids (SFAs) has shown benefits in diabetes and hypertension in humans (Schwingshackl and Hoffmann, 2012). Lamb meat is rich in SFAs, particularly palmitic and stearic acids, and MUFAs, mainly oleic acid (Enser et al., 1998). The enzyme stearoyl-CoA desaturase (SCD) plays an important role in ruminant species because it desaturates palmitic to palmitoleic acid, stearic to oleic acid (Enoch et al., 1976; Ntambi, 1999) and cis-vaccenic acid (VA) to CLA (Bauman et al., 1999), thus increasing the ratio of MUFAs to SFAs. In addition, the *SCD* gene encoding the SCD enzyme is located in a region where a positional quantitative trait locus (QTL) has been detected for the CLA:VA ratio in sheep milk (Carta et al., 2008), highlighting the beneficial role of *SCD* expression. Moreover, in sheep, the *SCD* gene harbours polymorphisms that have been shown to affect the fat content in milk, specifically the palmitoleic acid, linoleic acid, VA, SFA and MUFA contents and the n-6:n-3 and palmitoleic:palmitic ratios (García-Fernández et al., 2010). One of the SNPs associated with these traits is the rs412429481 SNP (FJ513370: g.31C>A), which is located in the promoter region of the ovine *SCD* gene and was previously described by Garcia-Fernandez et al. (2009). In bovines, several studies have shown associations between this gene and the fatty acid composition of both meat and milk (Taniguchi et al., 2004; Moioli et al., 2007). Expression of the *SCD* gene is regulated by the diet, especially by polyunsaturated fatty acids (PUFAs) of the n-6 and n-3 families, but also by hormones and environmental factors (Miyazaki et al., 2003). In ovine, grazing increases the amounts of CLA, total PUFAs (Scerra et al., 2007) and n-3 PUFAs (Dervishi et al., 2010) in lamb meat, a favourable change according to human dietary guidelines. It is well documented that, compared with forage, concentrate-based diets increase the

content of oleic acid in the tissues of cattle (Mitchell et al., 1991; Blanco et al., 2010) and sheep (Rowe et al., 1999; Lobón et al., 2017). Because of these data, we hypothesised that changes in diet or hormone treatments might increase the expression of the *SCD* gene, which in turn will decrease the SFA content while increasing both the oleic acid and cis-9, trans-11 CLA content of the meat, leading to an overall improvement in its nutritional quality. In sheep, only a few studies have investigated the nutritional regulation of gene expression in muscle (Vasta et al., 2009; Dervishi et al., 2011; Gonzalez-Calvo et al., 2017) but to the best of our knowledge, no studies linking DNA sequence variation to the responses to the nutrient responses have been carried out until now.

In the current study, we evaluated the functional impact of the rs412429481 (FJ513370: g.31C>A) SNP located at the promoter region of the *SCD* gene on the transcription rate of the gene as well as the influence of *SCD* genotypes on gene expression levels in regard to both nutrients response and the muscle type analysed (*longissimus thoracis* or *semitendinosus* muscles) in Rasa Aragonesa light lambs. Furthermore, we performed functional experiments *in vitro* to elucidate the molecular mechanism underlying the different gene expression levels found for the two alleles analyzsed.

2. Material and Methods

2.1. Animals

All experimental procedures, including animal care and slaughtering, were performed in accordance with the guidelines of the European Union and Spanish regulations for the use and care of animals in research and were approved by the Animal Welfare Committee of the research centre (protocol number 2009-01_MJT).

The study was conducted using the Rasa Aragonesa breed. The Rasa Aragonesa sheep breed belongs to the so-called entrefino type, which has short wool and wool fibres of medium thickness. These sheep are polled, have wool-less heads and are used mainly for meat. The area of distribution of the Spanish Rasa Aragonesa sheep is in northeast Spain. To study the putative functional impact of the rs412429481 SNP of the *SCD* gene on the transcription rate, two experiments were conducted with Rasa Aragonesa male lambs from weaning to slaughter at 22-24 kg live weight (LW):

- Experiment 1 (Exp. 1): Forty-four Rasa Aragonesa ewes and their spring single-born male lambs were allocated randomly to four treatment groups: alfalfa grazing, alfalfa grazing with a supplement for the lambs, indoor lambs with a supplement for the lambs with grazing ewes and drylot (Dervishi et al., 2010). The first two treatments were unweaned lambs grazing alfalfa, and the two last treatments were lambs weaned at day 45 and fed concentrates indoors. Differences were found in the MUFA and PUFA profiles between the grazing groups and the indoors concentrate groups. However, no differences were observed between the two grazing groups or the two indoor groups (Dervishi et al., 2010). Furthermore, cluster analysis of the expression profiles of 10 genes related to fatty acid metabolism (including the *SCD* gene) showed that 2 main clusters were formed according to the feeding system: grazing (alfalfa grazing and alfalfa grazing with a supplement for lambs) and indoor (indoor lambs with a supplement for lambs with grazing ewes and drylot) groups (Dervishi et al., 2011). Interestingly, both grazing groups showed lower levels of *SCD* expression than the indoor groups ($P < 0.05$). According to these results, the animals were divided into two groups for the analysis performed in this work: unweaned grazing alfalfa lambs (ALF; n=22: alfalfa grazing, alfalfa grazing with a supplement for the lambs) vs. weaned lambs fed concentrates indoors (IND; n=22: indoor lambs with a supplement for the lambs with grazing ewes and drylot). In the IND group, the lambs were weaned at 45 days old and had free access to concentrate. The slaughter weights were 23.38 ± 0.24

and 23.51 ± 0.36 for the ALF and IND groups, respectively (ALF lambs: 64.93 ± 4.74 days old; IND lambs: 77.84 ± 6.16 days old). The traditional farming systems in Mediterranean regions are extensive, semi-natural and natural grazing areas. However, there is a tendency to intensify the production of light lambs. Grass-based systems may be a good alternative to indoor lamb production systems because they use natural resources and provide the meat desired by consumers (Ripoll et la., 2014).

- Experiment 2 (Exp. 2): The study began at weaning (48.7 ± 0.21 days old) with an average LW of 18.5 ± 0.16 kg. Twelve lambs were fed *ad libitum* with a commercial concentrate without DL-α–tocopheryl acetate supplementation from weaning to slaughter, and the remaining lambs (n=36) were fed the same commercial concentrate but were supplemented with 500 mg of DL-α–tocopheryl acetate kg^{-1} concentrate for different finishing period lengths prior to slaughter. The length of time the supplemented concentrate was given ranged from 4 to 28 days (Ripoll et al., 2013). The slaughter weight and age were 23 ± 0.24 kg and 75.20 ± 1.84 days old, respectively.

The experimental procedures, composition of diets, management of the animals and sample details for each group are described in detail in Dervishi et al. (2010) and Ripoll et al. (2013) for Exp. 1 and Exp. 2, respectively.

In both trials, when the lambs reached 22–24 kg LW, they were slaughtered according to EU laws in the same commercial abattoir. Immediately after slaughter, samples of the *semitendinosus* (ST; Exp. 1 and Exp. 2) and *longissimus thoracis* (LT; Exp. 2) muscles were taken and frozen in liquid nitrogen until RNA and DNA isolation. The carcasses were chilled at 4°C for 24 h; then, the ST muscle and a piece from the 4th to the 6th lumbar *vertebrae* of the LT muscle were removed from the left halves of the carcasses.

2.2. Fatty acid analysis.

In both experiments the muscle fatty acid (FA) content was identified based on the protocol described in Dervishi et al. (2012). The individual FA contents were expressed as weight percentages (g/100 g of total FA). The proportions of total SFAs, MUFAs, PUFAs, n-3 PUFAs, n-6 PUFAs, PUFAs/SFAs and n-6/n-3 ratios were obtained from the individual fatty acid percentages.

2.3. SNP genotyping.

Genomic DNA was extracted from the animals in both studies using the SpeedTools DNA Extraction kit (Biotools, Madrid, Spain). The rs412429481 SNP (FJ513370: g.31C>A) is located in the promoter region of the ovine *SCD* gene and was previously described by Garcia-Fernandez et al. (2009). Genotyping of all animals was carried out using PCR-restriction fragment length polymorphisms (PCR-RFLPs) with the primers and amplifying conditions described by Garcia-Fernandez et al. (2009) (Table 1) and the *MnlI* restriction enzyme (New England Biolabs, Beverly, MA, USA). Fifteen μl of the PCR product was digested with 1 U of *MnlI* for 4 h at 37ºC in a total volume of 20 μl. The PCR-RFLP bands were visualised on 3% agarose gels stained with SYBR Safe (Invitrogen, Carlsbad, CA, USA).

2.4. Structural characterisation of the promoter region of the SCD gene.

Primers designed from sheep sequences NC_019479 and GQ904712 were used to amplify the promoter genomic region and partial exon 1 of the *SCD* gene (Table 1). PCR products containing the rs412429481 SNP were also used for the structural characterisation of this region. Genomic DNA (100 ng) of fifteen lambs with different genotypes for the rs412429481 SNP from Exp. 1 (7 CC, 6 CA, and 2 AA) was amplified

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in a final PCR volume of 25 µl, which contained 7.5 pmol of each primer, 200 nM dNTPs, 2.25 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100 and 1 U Taq polymerase (Biotools, Madrid, Spain). Standard amplification cycles were used. The PCR products were sequenced using an ABI Prism 3700 (Applied Biosystems, Madrid, Spain) and standard protocols. Homology searches were performed using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/) and CLUSTAL Omega software (https://www.ebi.ac.uk/Tools/msa/clustalo/).

2.5. In silico analysis of transcription factor binding and CpG islands.

To determine if nucleotide changes might result in changes in the binding ability of certain transcription factors, an *in silico* analysis of the surrounding sequence (plus and minus 30 nucleotides) was carried out using the Alibaba 2.1 search tool (http://www.generegulation.com/ pub/programs/alibaba2 /index.html). CpG island prediction was carried out with MethPrimer software (Li and Dahiya, 2002).

2.6. DNA methylation analysis of SCD.

The individual CpG methylation status of the promoter region harbouring the *SCD* rs412429481 SNP was analysed by bisulfite sequencing of the converted DNA. DNA was converted using a Methylcode Bisulfite conversion Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. A total of 24 DNA samples from Exp. 1 (6 CC, 8 CA and 1 AA from ST muscle of ALF treatment; 5 CC, 3 CA and 1 AA from ST muscle of IND treatment), and 19 DNA samples from Exp. 2 (7 CC, 7 CA and 5 AA from LT muscle from Exp. 2) were extracted and then bisulfite converted. Bisulfite-treated DNA was PCR amplified and sequenced using an ABI Prism 3700 (Applied Biosystems, Madrid, Spain). PCR was carried out in a final PCR volume of 25 µl, which contained 5 pmol of each primer, 2.0 mM MgCl2, 2.5 µl of 10X Certamp complex buffer

containing adjuncts and stabilisers and 1 U of Certamp complex enzyme mix (Biotools, Madrid, Spain). The primers for bisulfite sequencing PCR (BSP) and methylation-specific PCR (MSP) were designed using MethPrimer software (Li and Dahiya, 2002) and amplified a fragment of 199 bp, that included rs412429481 SNP. The primers used are described in Table 1. Standard amplification cycles were used. After sequencing the BSP or MSP products, the methylation patterns were determined by comparisons to untreated DNA.

2.7. Real-time quantitative PCR analysis (RT-qPCR).

Total RNA extraction (from approximately 500 mg of ST or LT muscle samples) and qPCR were carried out according to the methodology described in González-Calvo et al. (2014). The real time-PCR reaction was carried out in a 10 µL PCR total reaction mixture containing SYBR Green PCR Master Mix (Applied Biosystem, Madrid, Spain). Reactions were run in triplicate on an ABI Prism7500 platform (Applied Biosystem, Madrid, Spain) following the manufacturer's cycling parameters. To normalise the results of the *SCD* gene, 3 housekeeping (HK) genes were used for each muscle: *GAPDH*, *ACTB*, and *B2M* in the ST and *RPL19*, *B2M* and *YWHAZ* in the LT muscle. These HK genes were chosen because in previous studies, they were the most stably expressed genes in these tissues (Dervishi et al., 2011, 2012). Primer sequences for *SCD* and the HK genes are described in Dervishi et al. (2011, 2012). The corresponding mRNA levels were measured and analysed by their quantification cycle (Cq).

2.8. Reporter plasmids

Two luciferase reporter constructs for the A and C alleles were generated by cloning a region of the ovine *SCD* proximal promoter into the pGL3-Basic luciferase reporter

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vector (Promega, Madison, USA). We amplified a 595 bp DNA sequence of the ovine SCD proximal promoter (from −470 to +125, TSS defined as +1). The primers used for PCR introduced *HindIII* and *XhoI* restriction sites into the 5′ ends to enable directional cloning in pGL3-Basic (Table 1). PCR amplification was carried out in a volume of 25 μl, which contained 10 pmol of each primer, 200 nM dNTPs, 1.5 mM $MgSO_4$, 2.5 μl of 10X buffer, and 1 U KOD Hot Start DNA polymerase (Novagen, Merk Millipore, Madrid, Spain). Standard PCR cycles were performed according to the manufacturer's protocol. The PCR products were previously cloned in the pGEM®-T basic vector (Promega, Madison, USA) to remove polyA generated during PCR. Once excised from the pGEM®-T basic vector, the fragments were purified using a QIAquick gel extraction kit (Qiagen IZASA, Madrid, Spain) and cloned into the pGL3-Basic reporter vector previously digested with both *HindIII* and *XhoI* restriction enzymes. The cloned fragments of the *SCD* promoter and pGL3-Basic vector were digested with restriction enzymes, gel purified using the QIAquick gel extraction kit and bounded together with T4 ligase (Promega, Madison, USA).

Two other luciferase reporter constructs were generated by cloning the same DNA fragments (A and C alleles) synthesised as gene blocks (gBlocks; IDT, Sumalsa, Spain) with the *HindIII* and *XhoI* restriction sites. Cloning was performed in the same way as that for genomic DNA. The sequences of all plasmids were verified by Sanger sequencing.

2.9. Cell culture transfections and luciferase reporter assay

3T3L1 (mouse pre-adipocyte) cells were maintained in culture with Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA), supplemented with 2 mM L-glutamine and 10% newborn calf serum (NBCS). The cells were incubated at 37ºC in the presence of 5% CO_2. Twenty-four hours before transfection, 25,000 cells/well were

seeded into sixteen-well plates and grown to 70% confluence. 3T3L1 cells were transiently transfected into sixteen-well plates using Lipofectamine 3,000 transfection reagent (Thermo Fisher, Madrid, Spain) and Opti-mem media (Thermo Fisher, Madrid, Spain) according to the manufacturer's instructions. For transfections, 500 ng/well were used for each reporter vector. The Renilla gene (100 ng/well) served as an internal control for transfection efficiency. An empty pGL3-Basic vector was used as a negative control (500 ng/well). After 48 h, the cells were lysed with passive lysis buffer (Applied Biosystems, Thermo Fisher, Madrid, Spain), and luciferase activity was measured with the Dual-Glo luciferase assay system (Promega, Madison, USA) following the manufacturer's instructions. The intensities of Firefly and Renilla luciferase were measured using the Glomax20/20 Luminometer from Promega. In total, 7 transfections were performed: 4 transfections with luciferase reporter constructs with the ovine *SCD* proximal promoter using genomic DNA for each allele and 3 transfections for each allele with luciferase reporter constructs with the ovine *SCD* proximal promoter synthesised as gBlocks. Three independent experiments were carried out for each luciferase reporter construct and transfection.

2.10. Electrophoretic mobility shift assays (EMSAs).

To confirm the potential regulatory role of the rs412429481 SNP, the binding of nuclear proteins from C2C12 cells to both alleles was studied by EMSA. Additionally, SL-2 and SLC-pPAC-SP1 cells were used to check the binding to SP-1.

Nuclear extracts were prepared from C2C12 differentiated myotube cells as previously reported by Mozas et al. (2002). Nuclear extracts from Sp1-deficient Drosophila cell lines (SL-2) and from SL-2-transfected Drosophila cells with pPAC-SP1 (plasmid that expresses Sp1) were also used. SL-2 and SL-2-pPAC-SP1 cells were used as a control for SP1 expression.

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For assaying the binding of nuclear proteins to the rs412429481 SNP, 20 nt long double-stranded oligonucleotides with the sequence GGGGCTGCGG[C/A]GGCCAAACC were used. They were labelled at the 5' end with IRDye®680 (Tecknocroma, Madrid, Spain). Binding reactions were carried out as previously reported in Riancho et al. (2011). The DNA-protein complexes were subjected to gel electrophoresis on a 4% polyacrylamide gel in 0.25× Tris-borate-EDTA (TBE) buffer. The gel bands were analysed in an ODYSSEY infrared imaging system (Li-Cor Biosciences, Lincoln, NE, USA). Competition experiments were carried out using unlabelled A oligonucleotides (Riancho et al., 2011). The density of the gel shift bands was quantified using *ImageJ* software (NIH Bethesda, MA, USA). The EMSA experiment was replicated twice.

For supershift assays, 0.1 ng of polyclonal antibody specific for SP1 (Santa Cruz Biotechnology, CA, USA) was added to the reaction mixture with the labelled double-stranded oligonucleotides for the C and A alleles.

2.11. Statistical procedures.

Statistical analyses were performed using the SAS statistical package v. 9.3 (SAS Institute, Cary NC, USA). A P-value lower than 0.05 was considered statistically significant, and $0.05 \le P < 0.10$ was considered a trend.

2.11.1. Fatty acid analysis.

Statistical analysis of the fatty acid content was performed using different models for Exp. 1 and Exp. 2. In Exp. 1, the effects of the treatment on the fatty acid content were analysed by using the GLM procedure. In Exp. 1, the equation of the model used was as follows:

$$y_{im} = \mu + T_i + b_1(SA)_{im} + b_2(IMF)_{im} + e_{im}$$

where y_{im} is the fatty acid content corresponding to the mth animal feed with the ith treatment (ALF and IND); T_i is the fixed effect of the ith treatment; SA is the effect of the slaughter age; and IMF is the effect of the intramuscular fat included as a covariate.

In Exp. 2, the effect of the muscle on the content of each fatty acid was examined by using the mixed procedure:

$$y_{im} = \mu + S_i + b_1(SA)_m + b_2(IMF)_m + b_3(VE)_m + A_m + e_{im}$$

where y_{im} is the fatty acid content corresponding to the mth animal and to the ith muscle (ST and LT); S_i is the fixed effect of the ith muscle; SA is the effect of the slaughter age; IMF is the effect of the intramuscular fat; VE is the number of days of concentrate enriched with dl-α-tocopheryl acetate intake before the lambs reached their target slaughter weight included as a covariate; and the animal (A) and the residual (e) are included as random effects.

In both models, fatty acid content results were expressed as least square means (LSM) ± the standard error (SE) values and the differences were tested at a level of significance of 0.05 with the t statistic.

2.11.2. Analysis of expression results.

A statistical methodology to analyse differences in the expression rate of alternative genotypes of the polymorphism located at the gene promoter was described by Steibel et al. (2009). The equation for the mixed model used for the Exp. 1 was as follows:

$$y_{giomr} = \mu + TG_{gi} + MTG_{ogi} + b_1(SA)_{iom} + b_2(IMF)_{iom} + A_m + e_{giomr}$$

where y_{giomr} is the C_q (transformed data taking into account E < 2) obtained from the thermocycler software for the gth gene (*SCD* and the three HK genes) from the rth well (reactions were run in triplicate), corresponding to the mth animal and to the ith treatment (ALF and IND); TG_{gi} is the fixed interaction between the ith treatment and the gth gene (T is

the effect of the ith treatment and G is the effect of the gth gene); MTG_{oij} is the fixed interaction among the oth genotype (CC, CA and AA), the ith treatment and the gth gene (M is the effect the oth genotype of the *SCD* gene, T is the effect of the ith treatment and G is the effect of the gth gene); SA is the effect of the slaughter age included as a covariate; IMF is the effect of the intramuscular fat included as a covariate; A_m is the random effect of the animal from where the samples were collected ($A_m \sim N(0, \sigma^2_S)$); and e_{giomr} is the random residual. Gene-specific residual variance (heterogeneous residual) was also fitted to include variability among sample replicates for each gene within a treatment ($e_{giomr} \sim N(0, \sigma^2_{eig})$).

For Exp. 2, the two muscles were analysed separately. The mixed model fitted was as follows:

$$y_{gomr} = \mu + MG_{og} + b_1(SA)_m + b_2(IMF)_m + b(VE)_m + A_m + e_{gomr}$$

where y_{gomr} is the C_q (transformed data taking into account E < 2) obtained from the thermocycler software for the gth gene (*SCD* and the three HK genes) from the rth well (reactions were run in triplicate), corresponding to the mth animal; MG_{og} is the fixed interaction among the oth genotype (CC, CA and AA) and the gth gene (M is the effect of the oth genotype of the *SCD* gene, and G is the effect of the gth gene); SA (slaughter age), IMF (intramuscular fat) and VE (the number of days of concentrate enriched with dl-α-tocopheryl acetate intake) effects were included as covariates; A_m is the random effect of the animal from where the samples were collected ($A_m \sim N(0, \sigma^2_S)$); and e_{giomr} is the random residual. Gene-specific residual variance (heterogeneous residual) was also fitted to include variability among sample replicates for each gene within a treatment ($e_{giomr} \sim N(0, \sigma^2_{eig})$).

To test differences ($diff_{SCD}$) in the expression rate between the treatments and to obtain fold change (FC) values from the estimated TG and MTG (Exp. 1) and MG (Exp. 2)

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differences, the approach suggested in Steibel et al. (2009) was used to normalise the *SCD* expression to the three HK genes.

The significance of $diff_{SCD}$ estimates was determined with the *t* statistic. Additionally, asymmetric 95% confidence intervals (upper and lower) were calculated for each FC value using the standard error (SE) of $diff_{SCD}$

2.11.3. Luciferase assays.

The results from luciferase assays were analysed using the GLM procedure fitting a model in which the dependent variable was the relative luminescence unit (RLU) obtained for each genotype in each transfection. Transfection, genomic DNA or gBlocks luciferase reporter construct, replicate nested to genotype, and genotype were included in the model as fixed effects. Least square means, 95% confidence intervals and t tests for means comparison were calculated.

2.11.4. EMSA analysis.

The density of the gel shift bands was analysed using the GLM procedure. The effect of the gel was considered a fixed effect in the model. The results are presented as the mean ± standard deviation (SD). Least square means and t tests for mean comparisons were calculated.

3. Results

3.1. Fatty acid composition.

The effect of the feeding system on the fatty acid content in ST muscle of Exp.1 is reported in Supplementary Table S1. Lambs in the ALF group presented greater total SFA content ($P < 0.01$) and greater PUFA n-3 content ($P < 0.001$) but lower total MUFA content ($P < 0.01$) than their indoor fed-concentrate counterparts. Therefore, the n6:n3 ratio was lower in the ALF lambs than in the IND lambs ($P < 0.01$). No

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differences between the feeding systems were found for the total PUFA content and for total n-6 PUFAs (P > 0.05).

In Exp. 2, dl-α-tocopheryl acetate supplementation had no effect on the FA content of ST and LT muscles (P > 0.05; data not shown). The effect of the type of muscle (LT or ST) on the fatty acid content showed that the n-3 PUFA content was higher in ST muscle than in LT muscle (P < 0.001) due to the higher C20:5n-3, C22:5n-3 and C22:6n-3 contents (Supplementary Table S2). However, the MUFA content was higher in LT muscle than in ST muscle (P < 0.05) as a result of the higher of C18:1n-9 and C20:1n-9 contents observed in LT muscle.

3.2. Structural characterisation of the promoter region of the SCD gene.

The genotype and allele frequencies found for the rs412429481 SNP in animals from Exps. 1 and 2 are shown in Table 2. The genotype frequencies were in Hardy-Weinberg equilibrium. The promoter regions of 15 animals with different genotypes for the rs412429481 SNP (7 CC, 6 CA, and 2 AA) were sequenced to evaluate the presence of other polymorphisms linked to the rs412429481 SNP in this region. In total, 1844-bp and 183-bp sequences of the promoter region and exon 1, respectively, were analysed, but only the rs412429481 SNP was detected.

3.3. In silico analyses of transcription factor binding and DNA methylation analysis of SCD.

In silico analysis of transcription factor binding identified several overlapping putative transcription factors containing the rs412429481 SNP: Sp-1, AP-2 alpha, WT1 and NF1 (C allele) and Sp-1 and C/EBP alpha (A allele) consensus sites. CpG island prediction showed that the rs412429481 SNP was located in a 55-bp DNA fragment between two

CpG islands that were 193 and 377 bp. Furthermore, the C allele was predicted to be methylated.

After the bisulfite conversion of genomic DNA from both muscles, only the primers for bisulfite sequencing (BSP) designed to amplify the unmethylated DNA were able to produce an amplicon containing the rs412429481 SNP. Sequencing of this amplicon confirmed that this particular region was not methylated.

3.4. Expression analysies.

In Exp. 1, the expression of *SCD* was affected by slaughter age (P = 0.0003) and by the interaction between the SNP genotype and feeding system (P < 0.0001). *SCD* expression in IND lambs was 7.6

-fold higher than that in ALF lambs (P = 0.03) (Figure 1a). We excluded animals genotyped as AA in the analysis of *SCD* expression mediated by the rs412429481 SNP in Exp. 1 because we found only one animal per group genotyped as AA. In ALF lambs, there were no significant differences between the different genotypes. However, in the IND group, *SCD* expression was 5-fold higher in CA lambs (n =10) than in CC lambs (n =11; P = 0.04, Figure 1b).

In Exp. 2, the expression of *SCD* was affected by the slaughter age (P < 0.01) and the rs412429481 SNP in both muscles (P < 0.05). In ST muscle, *SCD* expression in CA lambs (n = 23) was 5-fold higher than that in CC lambs (n = 20; P = 0.014) (Figure 1b). In LT muscle, SCD expression in AA lambs (n= 5) was 24-fold higher than that in CA lambs (n = 22; P = 0.03, Figure 1b).

3.5. Luciferase assay

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We constructed luciferase constructs using both genomic DNA and synthetic DNA fragments (gBlocks). Due to extra mutations present in the PCR fragments amplified from genomic DNA containing the A variant of the SNP, these sequences were discarded. Then, synthetic sequences (gBlocks) had to be used instead. After sequencing the luciferase constructs, only the rs412429481 SNP was found. Across the different luciferase experiments performed, we consistently found that compared to the promoter containing the C allele, the promoter containing the A allele had a 23.9 % increase in its activity (Figure 2) (P = 0.057).

3.6. Electrophoretic mobility shift assays (EMSAs).

Electrophoretic mobility shift assays (EMSAs) suggested a difference in the ability of rs412429481 alleles to bind nuclear proteins from C2C12 cells. As shown in Figure 3, band 1 resulted from the incubation of C2C12 nuclear extracts with the labelled double-stranded oligonucleotide. The C allele (C2C12 lane with the C allele) showed stronger binding properties (P = 0.02) than the A allele (C2C12 lane with the A allele). The mean ratio (± s.d.) of the C allele gel shift band density to the A allele gel shift band density was 1.33 (±0.04).

In competition experiments, both allele shift band densities decreased with a 50-fold excess of unlabelled A allele oligonucleotides; these results indicated specific binding.

Band 1 did not disappear with incubation with the specific SP-1 antibody, and no anti-Sp1-specific supershifted complexes were observed in these experiments, indicating that the Sp1 nuclear protein was not present in the nuclear proteins. Furthermore, band 1 disappeared with the SL-2 extracts, and was replaced by a different band (2) (lanes SL-2 and SL2-pPAC-SP1 for each allele); in the SL2-pPAC-SP1 extracts, an additional band (3) was detected at different positions than 1,

suggesting that the protein that binds the oligonucleotide could be up-regulated by Sp-1.

4. Discussion

As expected, the fatty acid composition of the ST muscle was influenced by the feeding system. The greater content of n-3 PUFAs and C18:2 *cis*-9, *trans*-11 in ALF lambs than in IND lambs agrees with other previous studies in lambs (Dervishi et al., 2010; Aurousseau et al., 2004). Moreover, the oleic acid content was greater in IND lambs than in ALF lambs. Similar results have been reported in cattle (Mitchell et al., 1991; Blanco et al., 2010) and sheep (Rowe et al., 1999). In Exp. 2, we did not find a significant effect of dl-α-tocopheryl acetate (vitamin E) supplementation on the FA content. However, the effect of vitamin E on the FA profile is not yet clear, as conflicting results have been published (Berthelot et al., 2013; Liu et al., 2013). In the present study, it should be noted that the lack of effect can be related to the scarce fat depot observed in young lambs (younger than 90 days).

The rs412429481 polymorphism is located in the *SCD* promoter region at 255 nt from the *SCD* transcription start in putative trans-acting factor binding sites; therefore, it could be expected that alternative genotypes had an effect on gene expression. According to the results of the current experiments, we speculate that *SCD* expression could be regulated at two different levels: by the feeding system and by the rs412429481 SNP of the *SCD* gene. In grazing lambs, the genotype had no effect on gene expression, probably because the expression of the *SCD* gene is down-regulated by PUFAs (especially the n-6 and n-3 families), CLA, cholesterol and vitamin A (Ntambi, 1999; Landau et al., 1997; Choi et al., 2000). Grazing increases the amounts of PUFAs, CLA and n-3 PUFA (Dervishi et al., 2010; Aurousseau et al., 2004; Lobón et al., 2017), which may contribute to the relatively low expression of *SCD* (Dervishi et al.,

2010). In Exp. 1, greater n-3 PUFA content was found in the ALF group (Supplementary Table S1). A PUFA response region (PUFA-RR) containing sterol response element (SRE) and transcription factor nuclear factor-Y binding sites was described in the ovine *SCD* promoter by Zulkifli et al. (2010) at -362 to -401 from the start of transcription. These authors confirmed the *SCD* response to unsaturated fatty acids and that the PUFA-RR is required to elicit such a response. On the other hand, the rs412429481 SNP seems to modulate *SCD* expression during indoor concentrate feeding in both experiments and in both muscles, animals carrying the C- allele showed a lower expression rate than those carrying the A-allele. Similar PUFAs and n-3 PUFAs contents were found in the IND groups in both experiments (Supplementary Table S1 and Table S2); therefore, we speculate that in the IND groups, the expression of the *SCD* gene was not down-regulated by PUFAs but rather by the rs412429481 SNP. However, we found only two animals with the AA genotype in Exp. 1 (excluded from the gene expression analysis), and the significant results found in contrast with the AA genotype in Exp. 2 (AA-CA and AA-CC in LT muscle) might be misleading because the outcome relies on an unbalanced genotypes distribution (Table 2). Then, we aimed to confirm these results by luciferase assays and showed that the promoter containing the A allele had consistently higher activity than that containing the C allele.

Finally, the greater expression rate of the genotypes carrying the A- allele in concentrate-fed lambs was consistent with the EMSA results. The EMSA results suggested the presence of specific binding and allelic differences in the interaction with nuclear proteins. The assays showed that the oligonucleotide harbouring the C- allele of the rs412429481 SNP had higher binding affinity than that containing the A- allele. The *in silico* analysis of transcription factor binding sites suggested that the nucleotide change could alter the affinity of the SP-1 (A and C alleles), AP-2 alpha (C allele), WT1 (C allele), NF1 (C allele) and C/EBP alpha (A allele) nuclear proteins for the sequence including the rs412429481 polymorphism. The ubiquitous transcription factor Sp1 has

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been described as a transcriptional activator, but it can also act as a repressor of the expression of a vast number of genes involved in many cellular functions, such as differentiation, proliferation and apoptosis (Song et al., 2001; Doetzlhofer et al., 1999; Philipsen and Suske, 1999). Previous reports have identified Sp1 sites as regulatory DNA elements protecting against DNA methylation (Brandeis et al., 1994; Mummaneni et al., 1998; Boumber et al., 1998). Despite *in silico* predictions showing that this region could be methylated, methylation-specific PCR after the bisulfite conversion of DNA confirmed that the effect found on gene expression was not mediated by the methylation of the C allele or the predicted neighbouring CpG islands. In addition, a supershift EMSA assay revealed that the SP1 nuclear protein did not interact with this region of the *SCD* gene. CCAAT/enhancer binding protein α (C/EBP alpha) functions as a pleiotropic transcriptional activator of adipocyte genes during adipogenesis (Mandrup et al., 1997), including *SCD* (Ohsaki et al., 2007) in bovine adipocytes. AP-2 alpha is known to repress the expression of a number of genes, including C/EBP alpha (Jiang et al., 1998), Bcl-2 (Wajapeyee et al., 2006) and EGFR (Wang et al., 2006), but the mechanism of repression is unknown. Finally, the NFI recognition sequence was found in the promoter sequences of many cellular genes acting as transcription activators or repressors (Pjanic et al., 2011; Cooke and Lane, 1999). It is known that NFI occupies the promoters of many genes where it may bind synergistically with other transcription factors such as hepatocyte nuclear factor 1 alpha, oestrogen receptor, and Brg-associated factor (Satoh et al., 2005; Zhao et al., 2005). Here, two possible scenarios can be postulated. In the first one, the A allele could create a C/EBP binding protein motif stimulating gene expression, whereas the C allele would generate an AP-2 alpha site repressing gene expression or altering the affinity of transcription factors for their target motifs. The second possible scenario relies on the cooperative binding among different transcription factors and the fact that the rs412429481 polymorphism alters the relative affinity of one or more of these regulatory partners.

This polymorphism may serve as a potential genetic marker in breeding programs, but an appropriate sheep animal model is necessary to test the influence of rs412429481 on the meat FAs content.

5. Conclusion

The feeding system affects the fatty acid composition and *SCD* gene expression in the s*emitendinosus* muscle. Grazing lambs presented higher levels of n-3 PUFAs and C18:2c9t11 and lower *SCD* gene expression than indoor lambs. On the other hand, the *SCD* gene expression and luciferase assay data indicate that the rs412429481 SNP located in the *SCD* promoter modulates gene expression in both the *semitendinosus* and *longissimus thoracis* muscles of Rasa Aragonesa lambs. To the best of our knowledge, these are the first functional interactions shown between diet (grazing vs concentrate) and *SCD* genotype (rs412429481) in sheep.

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Conflicts of interest

We declare that we have no conflicts of interest.

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Table 1. Primer sequence, amplification size (bp), annealing temperature to amplify the *SCD* promoter region and partial exon 1 (fragments 1-3), primers for bisulfite sequencing PCR (BSP) (fragment 4) and methylation specific PCR (MSP) (fragment 5), and those used for obtaining promoter amplicon used for cloning (fragment 6). Fragment 3 was also used to genotype the rs412429481 SNP.

***SCD* fragment**	**Primers**	**Size (bp)**	**Tª (ºC)**
1	Forward: CACCTGCCCAGACTTCTCTC	757	58
	Reverse: CGTTGTTTTGGAATTGCCTT		
2	Forward: AAGGCAATTCCAAAACAACG	1066	55
	Reverse: TGCTGGGGATTTAAAGGCTA		
3	Forward: AAATTCCCTTCGGCCAATGAC	526	58
	Reverse: TCTCACCTCCTCTTGCAGCAA		
4	Forward: CGAGTTAATGGTAACGGTAGGACGA	199	55
	Reverse: AAAAAAAACAAAACTCCGAAACGTA		
5	Forward: TGAGTTAATGGTAATGGTAGGATGA	199	55
	Reverse: AAAAAAAACAAAACTCCAAAACATA		
6	Forward: CC<u>CTCGAG</u>TAAGAGAAGCCGAGGAGAAAGG (XhoI) [1]	611	55
	Reverse: CC<u>AAGCTT</u>CTCTCGGACACTGGGATCACTT (HindIII)		

[1] Restriction recognition sequence is underlined

Table 2. Numbers of animals (n) for each genotype and allelic frequencies (af) for the rs412429481 SNP of the SCD gene for experiments 1 (Exp. 1) and 2 (Exp. 2).

		Exp. 1			**Exp. 2**		
		CC	CA	AA	CC	CA	AA
Total							
	n	19	23	2	21	22	5
	af (C)		0.69			0.67	
Treatment[1]							
ALF	n	8	13	1			
	af (C)		0.66				
IND	n	11	10	1			
	af (C)		0.73				

[1] ALF: unweaned grazing alfalfa lambs (ALF); IND: weaned lambs fed concentrates indoors.

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Figure captions.

Figure 1. Differences in the expression rate between the treatment groups in Experiment 1 and among the genotypes in both experiments. Log2 fold change (FC) for the contrast between the IND and ALF treatments and between the CC and CA genotypes of the rs412429481 SNP in Experiment 1 (a) and among the alternative genotypes of the rs412429481 SNP in the longissimus thoracis and semitendinosus muscles in Experiment 2 (b). Segments indicate the 95% confidence interval of the fold change (FC_{UP}-FC_{LOW}). The significance level of the contrast is indicated over each bar (*P < 0.05, § P < 0.1). ALF: unweaned grazing alfalfa lambs (ALF); IND: weaned lambs fed concentrates indoors.

Figure 1.

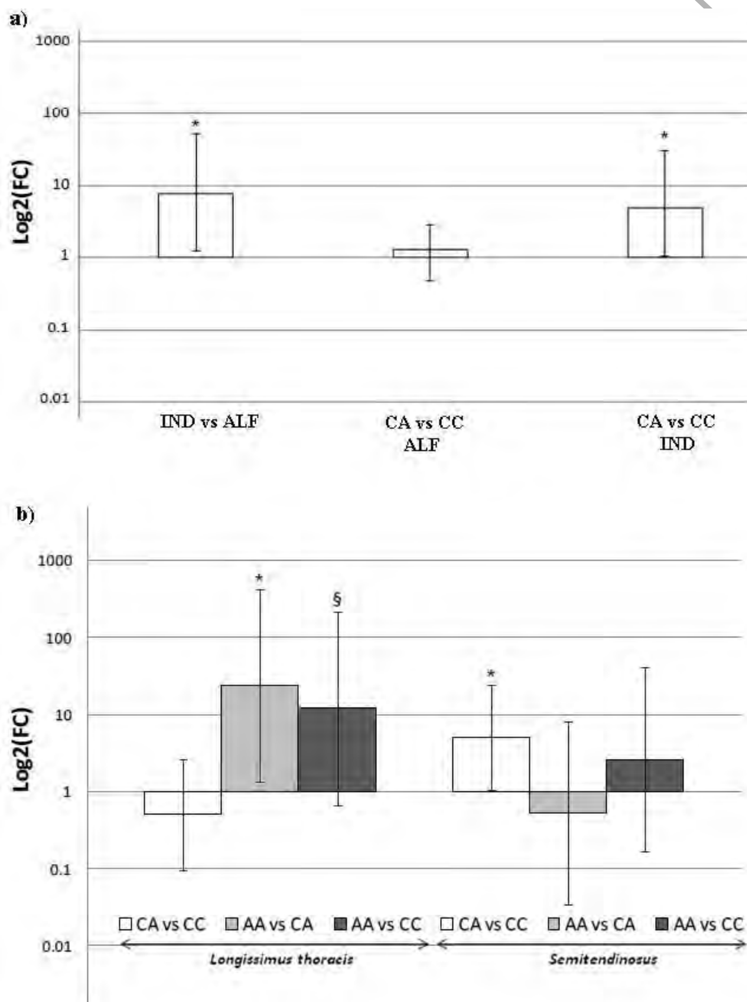

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Figure 2. Differences between least square means (lsmeans) in relative luminescence units (RLUs) for the contrast among constructs with alternative alleles of the rs412429481 SNP and the pGL3-Basic vector in the luciferase assay. Segments indicate the 95% confidence interval.

Figure 2.

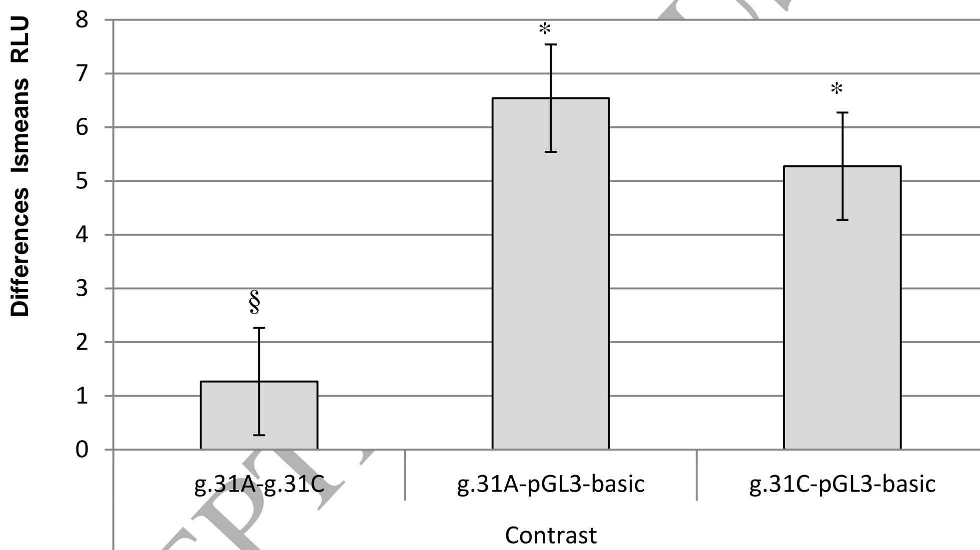

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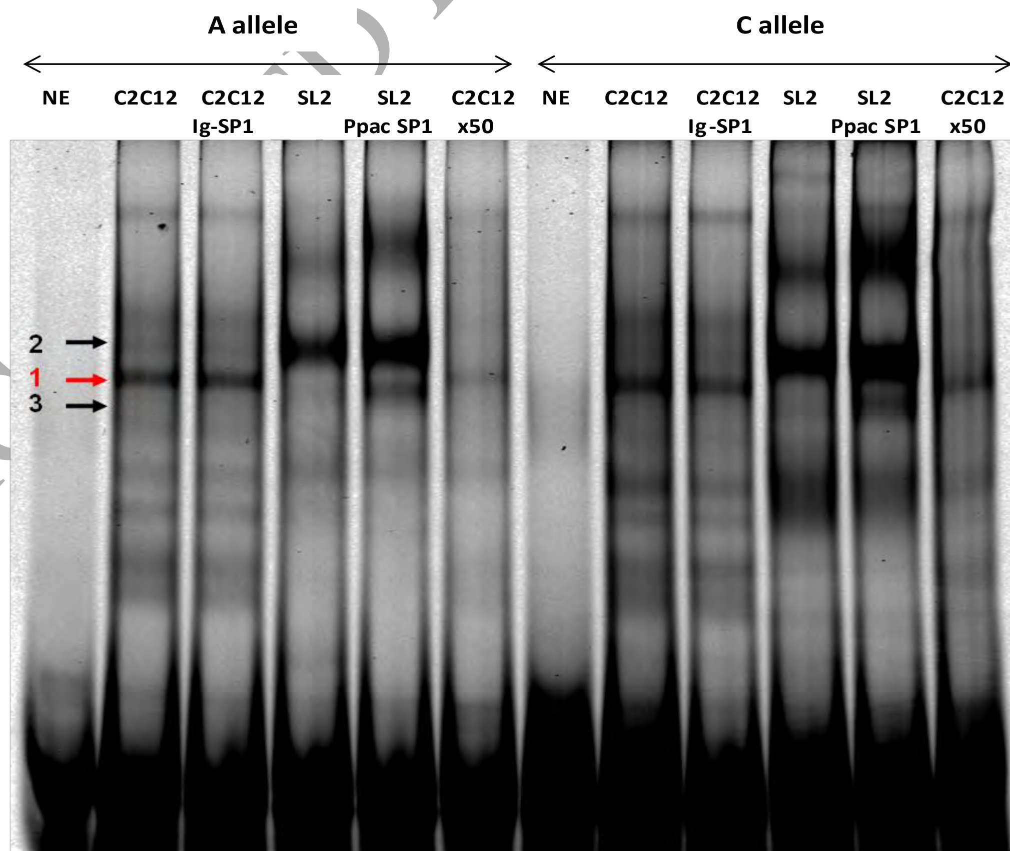
Figure 3. Electrophoretic mobility shif*t* assays (EMSAs). The six lanes on the left correspond to experiments performed with a labelled oligonucleotide specific for allele A of the rs412429481 SNP, whereas in the six lanes on the right, a labelled oligonucleotide specific for the C allele was used. NE indicates that no extract was added to the labelled probe. In the lanes labelled C2C12, SL2 and SL2 PpacSp1, the nuclear extracts from these cell cultures were added to labelled A- and C-specific probes. In the lanes labelled C2C12 50×, a 50-fold excess of an unlabelled A oligonucleotide was used to interfere with the formation of the complexes by either A-specific or C-specific probes. In lane C2C12 Ig-Sp1 a specific polyclonal antibody for the Sp1 transcription factor was used. The specific complex is indicated with an arrow.

Figure 3.



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