Differences in the expression of the *ASIP* gene are involved in the recessive black coat colour pattern in sheep: evidence from the rare Xalda sheep breed

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Summary

Here we have tested the hypothesis of association between different levels of *agouti signalling peptide* (*ASIP*) mRNA and the recessive black coat colour in the rare Xalda breed of sheep. To deal with this task, we first tested the possible action of both the dominant black *extension* allele (E^D) and a 5-bp deletion (X99692:c.100_104del; A^{del}) in the ovine *ASIP* coding sequence on the black coat colour pattern in 188 Xalda individuals. The E^D allele was not present in the sample and only 11 individuals were homozygous for the A^{del} *ASIP* allele. All Xalda individuals carrying the A^{del}/A^{del} genotype were phenotypically black. However, most black-coated individuals (109 out of 120) were not homozygous for the 5-bp deletion, thus rejecting the A^{del}/A^{del} genotype as the sole cause of recessive black coat colour in sheep. Differences in expression of *ASIP* mRNA were assessed via RT-PCR in 14 black-coated and 10 white-coated Xalda individuals showing different *ASIP* genotypes (A^{wt}/A^{wt} , A^{wt}/A^{del} and A^{del}/A^{del}). Levels of expression in black animals were significantly ($P < 0.0001$) lower than those assessed for white-coated individuals. However, the *ASIP* genotype did not influence the *ASIP* mRNA level of expression. The consistency of these findings with those recently reported in humans is discussed, and the need to isolate the promoter region of ovine *ASIP* to obtain further evidence for a role of *ASIP* in recessive black ovine pigmentation is pointed out.

Keywords agouti, coat colour, recessive black, RT-PCR, sheep.

The proportion of black lambs born causes substantial losses for the sheep industry (Fleet 2006). Consequently, identification of causes of recessive pigmented wool is of economic importance. Coat colour in mammals basically depends on the relative amount of the two basic types of tyrosine-derived melanin: eumelanin (black/brown) and phaeomelanin (yellow/red). The production of these pigments are controlled, in turn, by the *extension* (*E*) and *agouti* (*A*) loci, which are encoded by the *melanocortin-1 receptor* (*MC1R*) and *agouti signalling peptide* (*ASIP*) loci respectively (Bultman *et al.* 1992). Dominant black in sheep has been shown to be caused by an *extension* (E^D) allele carrying the p.M73K and p.D121N alterations in the coding sequence of the *MC1R* gene (Våge *et al.* 1999, 2003). However, the genetic basis of the

recessive coat colour pattern in sheep is still poorly understood.

It is usually accepted that the white-coated phenotype has phaeomelanin background from the dominant *white/tan* (A^{wt}) *agouti* allele, and that the most recessive ovine allele, *non-agouti* (A^a), results in a eumelanin (black/brown) coat pattern (Sponenberg 1997). In this respect, various analyses have reported linkage between non-dominant pigmentation in sheep and DNA markers on ovine chromosome 13, where the ovine *agouti* locus maps, indicating that the *ASIP* gene would be a positional candidate for recessive pigmentation in sheep (Parsons *et al.* 1999a; Beraldi *et al.* 2006). A 5-bp deletion (X99692:c.100_104del; note that the reference sequence X99692 is the *B. taurus* *ASIP* sequence, not the ovine *ASIP* sequence) has been described in exon 2 of the ovine *ASIP* gene, but its co-segregation with the recessive pigmentation phenotype in sheep has not been unambiguously determined so far (Smit *et al.* 2001, 2002). We have partially sequenced ovine *ASIP* exon 2 and intron 2, and the same deletion (c.100_104del) was found. This exon 2 sequence (EU274425, nt 1 to 135) showed 100%

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homology with the ovine cDNA agouti sequence EU057181 (Parsons *et al.* 1999b) except for the 5-bp deletion (c.100_104del) and 100% homology with the agouti-like sequence AY062211 (Smit *et al.* 2001). Even though the coding sequence of the *ASIP* gene is not the determinant of coat colour variation in all mammalian species (Royo *et al.* 2005; Drögemüller *et al.* 2006), the genetic scenario described by Smit *et al.* (2002) contrasts with that reported by Rieder *et al.* (2001) in horses in which a 11-bp deletion in exon 2 (AF288358:g.2174_2184del) is responsible for recessive black coat colour. Smit *et al.* (2002) suggested a model of segregation with two loci and two alleles per locus in which the wild-type allele (A^{wt}) is segregating from the ovine *agouti* locus and the deletion allele (A^{del}) is segregating from a separate, *agouti*-like locus in the ovine genome.

Following Våge *et al.* (1999, 2003), we have tested the presence of the E^D allele in four black-coloured sheep breeds: three Spanish breeds (Xalda, Black Merino and Black Castellana) and Karakul (Table 1). The only breed with no presence of the E^D allele was the Xalda sheep. The Xalda sheep is a rare breed in which black coat colour is traditionally segregating and, at present, is selected for by breeders (Álvarez Sevilla *et al.* 2003; Goyache *et al.* 2003). However, a substantial proportion of Xalda individuals are still white-coated, thus supporting the idea of recessive pattern(s) of inheritance of the trait. The Xalda individuals were genotyped for the presence of the A^{del} allele. A PCR protocol was designed using primers flanking the deletion (5'-GCACCTGAGGAAAAGCCAGAGATG-3' and 5'-CTTGATTCTCCAGAATTGTTCTG-3'), and alleles were determined based on its size (242 bp for the A allele, and 237 bp for the A^{del} allele) on an ALFexpressII automated sequencer (Amersham Biosciences). All Xalda individuals carrying the A^{del}/A^{del} genotype were phenotypically black. However, most black-coated Xalda individuals (91%) were not homozygous for the c.100_104del mutation, thus rejecting the A^{del}/A^{del} genotype as the sole cause of recessive black coat colour in sheep.

Recently, Voisey *et al.* (2006) have reported that differences in dark hair colour in humans are strongly associated with different levels of *ASIP* mRNA. We have obtained skin

samples from 24 (14 black-coated and 10 white-coated) Xalda individuals so as to assess possible differences in expression of the ovine *ASIP* mRNA (Fig. 1). Total RNA was extracted using a guanidine isothiocyanate-phenol technique (TRIzol® Reagent, GibcoBRL®), treated with DNaseI (TAKARA Bio®) and reverse-transcribed into single-stranded cDNA (First Strand cDNA Synthesis Kit for RT-PCR, AMV, Roche®). The mRNA levels of *ASIP* and *GAPDH* were measured by quantitative PCR with the iCycler Real Time PCR detection system (BioRad®) using the SYBR Green PCR Supermix (BioRad®). A 271-bp fragment of the ovine *agouti* gene was amplified using primers 5'-CACTCAGGCCTCC CAGGATGGATG-3' (forward) and 5'-CGTGCCACGTTCTTC ATCGGAG-3' (reverse). A 274-bp fragment of the ovine *GAPDH* gene was amplified using primers 5'-GTCACCAG GGCTGCTTTTAATTC-3' (forward) and 5'-CCAGCCTTCTC CATGGTAGTGA-3' (reverse). The *GAPDH* transcript was used as endogenous control following Drögemüller *et al.* (2006) for pig *agouti* skin expression. Each individual cDNA sample was amplified in duplicate and the real-time assay was repeated twice. Expression was estimated by the $\Delta\Delta Ct$ method (Drögemüller *et al.* 2006).

Level of expression was treated as a continuous variable and statistically analysed using PROCGLM of the SAS/STAT® package, fitting a linear model that included as fixed effects the phenotype (with two levels: white and black) and the *agouti* genotype (with three levels: A^{wt}/A^{wt} , A^{wt}/A^{del} and A^{del}/A^{del}). The phenotype was significantly associated with *ASIP* mRNA levels ($P < 0.0001$; least square means of 735.17 and 13.60 for white and black individuals respectively) whilst genotype was not ($P = 0.531$). Voisey *et al.* (2006) reported that differences in expression of the human *ASIP* mRNA are associated with a polymorphism in the 3'UTR region of *ASIP*, with unknown functionality, which could affect mRNA stability or could be in linkage disequilibrium with a promoter polymorphism affecting transcription. A similar hypothesis can be proposed as a partial explanation of the recessive black coat colour pattern in sheep.

It is well known that various segregation patterns affecting the same or different loci can produce similar

Table 1 Absolute frequencies of the *extension* and *agouti* genotypes considering the dominant (E^D) and wild-type (E^+) *MC1R* alleles and the wild-type and 5-bp deletion (A^{wt} and A^{del} respectively) *ASIP* alleles in three Spanish sheep breeds in which the black coat is segregating (Xalda) or fixed (Black Merino and Black Castellana), as well as in the Karakul breed.

Breed	n	Extension locus genotype			Agouti locus genotype		
		E^D/E^D	E^D/E^+	E^+/E^+	A^{wt}/A^{wt}	A^{wt}/A^{del}	A^{del}/A^{del}
Black-coated Xalda	120	0	0	120	52	57	11
White-coated Xalda	68	0	0	68	20	48	0
Karakul	5	5	0	0	5	0	0
Black Merino	12	12	0	0	1	11	0
Black Castellana	30	30	0	0	12	17	1

ASIP, agouti signalling peptide; *MC1R*, melanocortin-1 receptor.

Figure 1 Differences in *agouti signalling peptide* (ASIP) skin mRNA expression in 24 Xalda individuals. Black-coated phenotypes are in black bars whilst white-coated phenotypes are in grey bars. Numbers on the top of the bars mean different ASIP genotypes: 0 (A^{wt}/A^{wt}); 1 (A^{wt}/A^{del}); and 2 (A^{del}/A^{del}).

phenotypes in sheep (Sponenberg 1997) and, for instance, a white-coated phenotype can be produced by the action of *agouti* alleles but also by the *spotting* locus. In contrast with previous work (Smit *et al.* 2001), here we have determined that the dominant black allele (E^D) is not affecting our results and, from our data, we cannot reject that the homozygous A^{del}/A^{del} genotype could cause by itself recessive black-coated phenotype in sheep. However, this genotype cannot be the sole cause of the black recessive pigmentation pattern, and differences in expression of ASIP mRNA are also associated with this phenotype regardless of *agouti* genotype. These results also suggest that there may only be a single *agouti* locus segregating in the Xalda breed. As in humans, the promoter region of ovine ASIP has yet to be isolated and may provide further evidence for a role of ASIP gene in recessive black ovine pigmentation.

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