Correlation of single nucleotide polymorphisms in the melanocortin-1 receptor gene with colour in *Vicugna pacos* (Alpaca)

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Summary

The aim of this study was to determine if any correlation exists between *MC1R* polymorphisms, and skin and fibre colour in alpacas. Primers capable of amplifying the entire alpaca *MC1R* were designed from a comparative alignment of *Bos taurus* and *Mus musculus MC1R* gene sequences. The complete *MC1R* of 36 alpacas exhibiting a range of fibre colours, and which were sourced from farms across Australia, was sequenced from PCR products. Twenty one single nucleotide polymorphisms were identified within *MC1R*. Two of these polymorphisms (A82G and C901T) have the potential to reduce eumelanin production by disrupting the activity of MC1R. No agreement was observed between fibre colour alone, and *MC1R* genotype in the 36 animals in this study. However, when the animals were assigned to groups based on the presence or absence of eumelanin in their fibre and skin; only animals that had at least one allele with the A82/ C901 combination expressed eumelanin. We propose that A82/ C901 is the wildtype dominant "*E*" MC1R allele, while alpacas with either G82/ T901 or G82/ Y901 are homozygous for the recessive "*e*" MC1R allele and are therefore unable to produce eumelanin.

Keywords: alpaca, melanocortin 1 receptor, *MC1R*, pigmentation, melanin, single nucleotide polymorphisms.

Introduction

The Australian alpaca fibre industry has progressed in the last three decades from a breeding phase to a commercial fibre industry. The current focus of the industry is on optimising fibre production to meet market demand (Australian Alpaca Fleece Ltd 2004; Frank *et al.* 2006; McGregor 2006). The price and desirability of Alpaca fibre is governed by characteristics including fibre type, length, diameter, evenness, yield and colour (Frank *et al.* 2006). Colour is an important fibre characteristic because of its influence on the potential applications of the end product. There is currently no definitive model to explain the mechanisms that govern fibre colour inheritance in alpacas, therefore breeding programs are not efficient in selecting for colour (McGregor 2006). Current colour breeding strategies use the fibre colour of the parents to predict breeding outcomes (Australian Alpaca Fleece Ltd 2004; Paul 2006). However, phenotype can be a poor indicator of genotype because of the complex nature of pigment regulation in mammals (Rees 2003; Hoekstra 2006). Fibre phenotype is the outcome of the combined effect of numerous genes (Furumura *et al.* 1996; Sponenberg 2001). Exactly which genes and alleles are important in alpaca fibre colour remains to be determined. The ability to determine the mechanisms of fibre colour inheritance in alpacas would be highly valuable to the industry because it would facilitate more precise selection and breeding for desired colours.

Pigmentation mechanisms in mammals have been extensively studied with over 100 genes known to be involved in the pigmentation process (Guibert *et al.* 2004; Hoekstra 2006; Ishida *et al.* 2006). These mechanisms are known to be highly conserved among mammals and therefore information from well-characterised species can serve as a suitable basis for predicting mechanisms involved in alpaca coat colour inheritance (Rees 2003; Hoekstra 2006). Mammalian coat colour is predominantly

dependent on the amount, type and distribution of melanin in hair follicles (Furumura *et al.* 1996; Rees 2003). Mammals are capable of producing two chemically distinct melanin types known as eumelanin and pheomelanin, which are responsible for black to brown, and red to yellow pigment respectively (Smith *et al.* 2001; Oyehaug *et al.* 2002). While pigmentation is a complex process known to be influenced by a large number of genes, the primary regulation of pigment type is controlled by a key gene known as the Melanocortin-1 receptor (*MC1*; Sturm *et al.* 2001; Slominski *et al.* 2004; Rouzaud & Hearing 2005).

MC1R is a membrane bound G-protein coupled receptor (GPCR) expressed on the surface of melanocytes (Scott *et al.* 2002; Newton *et al.* 2005). GPCRs are an extensive family of cell surface proteins which display a common molecular structure consisting of five types of domains; an extracellular N terminus and seven transmembrane fragments connected by three extracellular and three intracellular loops, and an intracellular C terminus (Schoneberg *et al.* 2004; Sanchez-Mas *et al.* 2005; Tao 2006). These domains have roles that are vital for the functionality of the receptor and therefore polymorphisms located in important domains may affect pigmentation.

The association between polymorphisms within *MC1R* and pigment variation has been extensively studied and documented in a range of mammals including mice (Hoekstra *et al.* 2006), pigs (Kijas *et al.* 1998), cows (Rouzaud *et al.* 2000), dogs (Everts *et al.* 2000), foxes (Vage *et al.* 2005), horses (Marklund *et al.* 1996) and humans (Frandberg *et al.* 1998; Grimes *et al.* 2001). The objective of this study was to characterise *MC1R* in alpacas and to investigate the association of polymorphisms within *MC1R* with colour.

Materials and methods

Animals and DNA extraction

Blood samples were collected from 36 alpacas (31 huacaya, 5 suri; comprising 8 black, 2 bay, 4 grey, 3 dark brown, 4 dark fawn, 3 fawn, and 12 white animals). Samples were collected from animals bred in Western Australia, New South Wales and Victoria, Australia. Genomic DNA was extracted from 200 μl of EDTA-anticoagulated blood using the DNeasy tissue kit (Qiagen) according to the manufacturer's instructions.

Amplification, cloning and sequencing of alpaca MC1R

Alpaca *MC1R* Primers MC1R1-F and MC1R2-R (Table 1) were designed to hybridise to conserved regions of the cow (*Bos taurus*) and mouse (*Mus musculus*) *MC1R* sequence (NCBI accession numbers NM_174108 and NM_008559 respectively), and were used to amplify the complete alpaca *MC1R* coding region.

All polymerase chain reactions (PCR) used an Eppendorf Mastercycler, in a 10μl reaction containing 67mM Tris.HCl (pH8.8), 16.6mM $[NH_4]_2SO_4$, 0.45% v/v Triton X-100, 0.2mg/ml Gelatin, 0.2mM dNTPs (Fisher Biotech), 2μM each of forward and reverse primer, 1 U of *Taq* DNA polymerase (Fisher Biotec), 1.5mM $MgCl_2$, and 100ng of genomic DNA. Thermal cycles were: initial denaturation at 95°C for 5 min, followed by 30 cycles, each consisting of 94°C for 30s, 58°C for 30s and 72°C for 1 min and 30s; with a final extension at 72°C for 10 min. Amplified DNA was analysed by electrophoresis on 1.5% (w/v) agarose gel in TAE buffer, stained with ethidium bromide and visualised by UV transillumination.

PCR products from each animal were purified using the UltraClean PCR Clean-up Kit (Mo Bio). DNA was cloned using the pGEMTeasy cloning kit (Promega), and each

clone was sequenced using both M13F and M13R primers (Table 1) with Big Dye Terminator Technology (Applied Biosystems) and analysed on an 3730 DNA analyser (Applied Biosystems).

The sequences obtained from the two cloned PCR fragments were used to design alpaca-specific primers, MC1R3-F and MC1R4-R (Table 1), which were subsequently used to amplify *MC1R* from 36 animals using the PCR conditions described above. Amplification of *MC1R* from each animal was performed in five independent 10 μl reactions which were pooled for product purification and sequencing. Sequencing reactions were performed as described above except using four primers for each product: MC1R3-F and MC1R4-R, and two alpaca-specific internal primers, MC1R5-F and MC1R6-R (Table 1). Complete MC1R sequences for each animal were compiled into contigs using Vector NTI software (Invitrogen), and compared with genes and proteins from other species by NCBI BLASTn and BLASTx protocols.

Results

Alpaca *MC1R* sequence

An NCBI BLAST search confirmed homology with *MC1R* of other species (GenBank accession number EU135880). Sequence similarity with alpaca was: pig and sheep 88%, goat 87%, cow 86%, human 85%, horse 84% and mouse 81%. Twenty one single nucleotide polymorphisms (SNP) were identified within the MC1R coding regions in the 36 animals. Twelve polymorphisms occurred in only one animal (C72G, A85T, G112A, G140C, A265G, T296C, T299C, C367T, T379C, T383C, A595G, and A730G) and two occurred in only two animals (C92T and T587C). However, seven of the SNP were present in numerous animals (Table 2).

Translation of the *MC1R* sequence revealed an open reading frame of 317 amino acids. Four of the seven common SNP caused no amino acid change (D42D, N118N, L206L and E311E) while the remaining three resulted in amino acid substitutions (T28A, G126S and R301C). The alpaca MC1R protein was most similar to sheep MC1R (88%), followed by cow and cat (87%), pig (85%) and horse and dog (84%). No correlation was observed between fibre colour alone, and MC1R genotype in the 36 animals studied. However, when the animals were assigned to groups based on the presence or absence of eumelanin in fibre and skin; only animals that had at least one allele with the A82/ C901 (Table 2) combination expressed eumelanin. Animals that did not have an A82/ C901 combination (i.e. were G82/ T901 or G82/ Y901 genotypes) expressed only pheomelanin (Table 2).

Discussion

This study identified a putative wildtype alpaca *MC1R* and a non-functional recessive variant. Two of the SNP identified in this study were correlated with the presence (A82/ C901) or absence (G82/ T901 or G82/ Y901) of eumelanin in skin and fibre. The animals expressing pheomelanin-only are hypothesised to have the genotype *ee* representing the homozygous recessive genotype at *MC1R*, while the eumelanic animals are proposed to have the genotypes *EE* (homozygous wild-type) or *Ee*, which both allow normal eumelanin expression. Breeding records (where available) supported this observation. For example, the three black animals that were heterozygous *Ee* each had one black and one pheomelanic parent (data not shown). The presence of pheomelanic animals with apparently similar phenotype, but different genotype, is explained when the *Agouti* gene is considered. *Agouti* is a colour regulation gene that has an epistatic relationship with *MC1R* (Furumura *et al.* 1996; Lin & Fisher 2007). Expression of *Agouti* alleles requires the presence of a fully

functioning wild type *MC1R* (Hart 2001; Kerns *et al.* 2003; Paul 2006). In many species variant *Agouti* alleles cause a range of colours from completely eumelanic to completely pheomelanic and many intermediate patterns (Rieder *et al.* 2001; Voisey & van Daal 2002; Kerns *et al.* 2004; Girardot *et al.* 2005). We suggest that a similar effect exists in alpacas, in that white fibred black-skinned alpacas are genetically different from white fibred pink-skinned alpacas. Three of the animals had indeterminate eumelanin status (shown as "?" in Table 2), due to difficulty in assigning a definite phenotype. Breeding records for these animals were either unavailable or not definitive, but those that were available supported the hypothesis that the genotype assigned to them was accurate.

It is difficult to determine the effect of SNP on the functionality of MC1R without functional analysis of the alternative proteins, and there are no previous reports of either a T28A or an R301C polymorphism in MC1R of any species. The R301C polymorphism occurs in the c-terminus of the protein. Exchanging an arginine for a cysteine may affect the structure of the MC1R C-terminus. However determining if this is the case is particularly difficult given the region in which the polymorphism occurs. Many SNP affect protein function, yet structural modelling is difficult because of the inability of modelling programs to adequately detect the effect of an SNP on protein structure and/or expression (Stitziel *et al.* 2003). This is especially the case where the polymorphism occurs in the C-terminal loop of the amino acid chain, which has a tendency to be flexible and difficult to model (Krystek *et al.* 2006).

The C-terminus in MC1R is a functionally important domain involved in interactions with the ligand-receptor complex with G-proteins, placement of the receptor within the membrane, and providing signals for intracellular trafficking (Schoneberg *et al.* 2004; Garcia-Borron *et al.* 2005; Sanchez-Mas *et al.* 2005). Polymorphisms in this

domain were reported to impair receptor function severely (Everts *et al.* 2000; Garcia-Borron *et al.* 2005; Sanchez-Mas *et al.* 2005). A non-functional or reduced function MC1R would result in exclusive, or increased, production of pheomelanin, respectively (Newton *et al.* 2000; Logan *et al.* 2003; Hoekstra 2006).

The R301C polymorphism encodes two alternate amino acids which have different properties. Arginine is a polar, positively charged amino acid and cysteine, while still polar, has no charge. Both are hydrophilic amino acids (Stoker 2001). Changing from a positively charged amino acid to a neutral amino acid could prevent proper interaction of the receptor with G-proteins and result in a non-functional receptor.

The T28A polymorphism occurs in the extracellular loop of the N-terminus of MC1R. Some residues in this domain have been shown to have functional significance (Garcia-Borron *et al.* 2005). Alanine is a non-polar, hydrophobic amino acid while threonine is a polar hydrophilic amino acid (Stoker 2001). Their different properties may not necessarily indicate a structural change in MC1R. It is probable that R301C is more likely than T28A to completely abolish receptor function.

There is evidence to suggest that some MC1R variants are required for, but are not alone sufficient to cause, a change in pigmentation (Voisey *et al.* 2001). This is demonstrated in humans with red hair alleles, for which there is strong evidence that a combination of polymorphisms, rather than the effect of a single polymorphism, results in diminished activity of the receptor (Sturm *et al.* 2001; Carroll *et al.* 2005; Makova & Norton 2005). Different combinations of polymorphisms can therefore have either a strong or weak effect on the phenotype, resulting in the large variation seen between red haired individuals (Carroll *et al.* 2005; Makova & Norton 2005). The evidence suggests that the polymorphisms identified in this study have a cumulative effect on the functionality of MC1R.

The G126S polymorphism occurs in the central portion of the third transmembrane fragment. This results in the substitution of a non-polar glycine for a polar uncharged serine. We found no correlation of any phenotypic trait with alleles of this polymorphism (Table 2). Polymorphisms occurring in the third transmembrane region, which have functional significance, are most often confined to the boundary between the transmembrane fragments and the extracellular loops (Robbins *et al.* 1993; Vage *et al.* 1999). The majority of phenotype-affecting mutations located in these domains are activating-mutations (Robbins *et al.* 1993; Vage *et al.* 1999). The G126S polymorphism is located in the central portion of the domain, and we have found no reports of this SNP being associated with coat variation documented in any other species. Therefore it is not probable that G126S affects alpaca colour.

Single nucleotide polymorphisms have been estimated to occur at a rate of approximately 1 per 1000 bp in the mammalian genome, with the majority occurring in intronic DNA (Stitziel *et al.* 2003). This study reports the identification of seven SNP within a 951bp gene. This is consistent with the high variability demonstrated in the MC1R gene in other species (Jimenez-Cervantes *et al.* 2001; Majerus & Mundy 2003; FitzGerald *et al.* 2006).

This study examined animals representing eight of the20-plus recognised fibre colour phenotypes (Cecchi *et al.* 2004; Paul 2006) and both coat types (Suri and Huacaya). Within both the colour subgroups there was no difference in *MC1R* genotype between white, fawn, bay and black animals, nor between Suri and Huacaya coat types. It is probable that other genes are acting to modify coat colour. Pigmentation is a complex process with the potential for colour to be affected at many stages in the process (Rees 2003; Slominski *et al.* 2004). Some of the genes that affect colour in other species

such as *Slc7a11*, *Rab27a*, *Myosin Va* and *Mlph* (Chintala *et al.* 2005; Hearing 2005; Hoekstra 2006; Ishida *et al.* 2006) may cause similar colour variation in alpacas. The use of molecular data, combined with extensive phenotype records, and application of Mendelian principles, appears to be a successful approach to investigate colour genetics in alpacas. This approach allowed us to propose a mechanism that explained some of the colour variation seen in alpacas, where molecular data or fibre colour records alone would not have allowed us to make these correlations between genotype and phenotype.

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Table 1 Primers used in this study

Primers	Sequence
MC1R1-F:	CTGGCACCACGGAGCTTG
MC1R2-R:	CTCCTCAGCCTGCTCCATTC
MC1R3-F:	GGGAGAAGGTGAGTGTGAGG
MC1R4-R:	GCTCTTCCTGGAGATTCGTG
MC1R5-F:	CATGGTGTCCAGCCTCTGCT
MC1R6-R:	CCACCCAGATGGCCGCGATG
M13-F:	CCCCAGGGTTTTCCCAGTCACGAC
M13-R:	TCACACAGGAAACAGCTATGAC

Table 2 The colour phenotype and *MC1R* genotypes of the alpaca samples examined in this study. *"E"* denotes the proposed wild type allele and *"e"* denotes the proposed recessive alleles at *MC1R*. SNP in bold are those from which the genotype is derived.

SNP Genotype									
82 (T28A)	126 (D42D)	354 (N118N)	376 (G126S)	618 (L206L)	**901 (R301C)**	933 (E311E)	**Fibre Colour**	**Eumelanin Present***	**MC1R alleles**
A	T	T	G	G	**C**	G	fawn	Y	*EE*
A	T	T	A	G	**C**	G	white	Y	*EE*
A	T	T	G	G	**C**	G	fawn	Y	*EE*
A	T	T	G	G	**C**	G	dk fawn	Y	*EE*
A	T	T	A	G	**C**	G	black	Y	*EE*
A	T	T	R	G	**C**	G	black	Y	*EE*
A	T	T	A	G	**C**	G	silvergrey	Y	*EE*
A	T	T	A	G	**C**	G	dk brown	Y	*EE*
A	T	T	A	G	**C**	G	med grey	Y	*EE*
A	T	T	A	G	**C**	G	black	Y	*EE*
A	T	T	A	G	**C**	G	black	Y	*EE*
A	T	T	A	G	**C**	G	black	Y	*EE*
A	T	T	A	G	**C**	G	white	?	*EE*
R	Y	Y	R	R	**Y**	R	bay	Y	*Ee*
R	Y	Y	R	R	**Y**	R	dk fawn	Y	*Ee*
R	Y	Y	R	R	**Y**	R	black	Y	*Ee*
R	Y	Y	G	R	**Y**	R	white	Y	*Ee*
R	Y	T	R	R	**Y**	R	bay	Y	*Ee*
R	Y	Y	G	R	**Y**	R	black	Y	*Ee*
R	Y	Y	A	R	**Y**	R	med fawn	Y	*Ee*
R	Y	T	R	R	**C**	R	bay	Y	*Ee*
R	Y	Y	R	R	**C**	R	black	Y	*Ee*
G	C	C	G	A	**T**	A	dk fawn	?	*ee*
G	C	C	G	A	**Y**	A	dk fawn	?	*ee*
G	C	C	G	A	**T**	A	white	N	*ee*
G	C	C	G	A	**T**	A	med fawn	N	*ee*
G	C	C	G	A	**T**	A	white	N	*ee*
G	C	C	G	A	**T**	A	fawn	N	*ee*
G	C	C	G	A	**T**	A	white	N	*ee*
G	C	C	G	A	**T**	A	white	N	*ee*
G	C	C	G	A	**T**	A	white	N	*ee*
G	C	C	G	A	**T**	A	white	N	*ee*
G	C	C	G	A	**T**	A	white	N	*ee*
G	Y	C	G	A	**T**	T	rosegrey	N	*ee*
G	C	Y	G	A	**Y**	A	white	N	*ee*
G	C	C	G	R	**Y**	R	white	N	*ee*

* Y: eumelanin is present in either skin or fibre or both. N: No eumelanin is present in skin or fibre. ?: eumelanin status is unable to be determined.