Archiv Tierzucht 56 (2013) 5, 42-49

Short communication

Investigation of the premelanosome protein *(PMEL or SILV)* gene and identification of polymorphism excluding it as the determinant of the dilute locus in domestic rabbits *(Oryctolagus cuniculus)*

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Abstract

After the rediscovery of the Mendel's laws, the domesticated European rabbit (*Orycolagus cuniculus*) has been the objective of pioneering studies on coat colour genetics. However, despite the early role of this species in defining genetic mechanisms determining this phenotypic trait, only recently a few loci have been characterized at the molecular level analysing also in rabbits genes already shown to affect coat colour in mice. We herein investigated the rabbit premelanosome protein (*PMEL*) gene, also known as melanocyte protein Pmel 17 (*PMEL17*) or silver (*SILV*), as mutations in the homologous gene in mice and other species produce phenotypic effects similar to what is observed in the dilute coat colour in rabbit. The rabbit dilute locus is determined by a recessive coat colour mutation that dilutes the black to blue (grey) interacting with the basic colours influenced by the agouti and extension loci. To investigate this candidate gene, we isolated and sequenced cDNAs as well as portions of intronic and exonic regions of the *PMEL* gene in several rabbits with different coat colours and identified single nucleotide polymorphisms, including several missense mutations. One polymorphism, positioned in intron 7, was genotyped in a family in which there was segregation of the dilute coat colour. The results excluded *PMEL* as the causative gene for the dilute locus in rabbits, shortening the list of candidate genes that should be analysed to identify the mutation determining this phenotypic trait.

Archiv Tierzucht 56 (2013) 5, 42-49
doi: 10.7482/0003-9438-56-005

Received: 3 November 2011
Accepted: 5 June 2012
Online: 8 February 2013

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Keywords: candidate gene, coat colour, premelanosome protein, rabbit, SNP

Abbrevations: cDNA: complementary DNA, DNA: deoxyribonucleic acid, KLD: Kringle-like domain, NTR: N-terminal region, PCR: polymerase chain reaction, PKD: polycystic kidney disease, PMEL: premelanosome protein gene, PMEL17: melanocyte protein Pmel 17, RNA: ribonucleic acid, SNP: single nucleotide polymorphism

Introduction

After the rediscovery of the Mendel's laws, the domesticated European rabbit (*Orycolagus cuniculus*) has been the objective of pioneering studies on coat colour genetics based mainly on fancy breeds/lines (Castle 1905, Castle 1930, Robinson 1958).

Despite the early role of the rabbit in defining genetic mechanisms determining this phenotypic trait, only recently a few loci have been characterized at the molecular level analysing also in rabbits (Aigner *et al.* 2000, Fontanesi *et al.* 2006, 2010a, 2010b) genes already shown to affect coat colour in mice (Kwon *et al.* 1987, Bultman *et al.* 1992, Robbins *et al.* 1993). Among these loci, *agouti* and *extension* control the production and relative amount of eumelanin (black/brown pigments) and pheomelanin (red/yellow pigments). They encode the agouti signalling protein (*ASIP*) and melanocortin 1 receptor (*MC1R*) genes, respectively (Bultman *et al.* 1992, Robbins *et al.* 1993).

Several other coat colour loci remain to be characterized at the DNA level in the rabbit. Among them, the dilute locus is determined by a recessive coat colour mutation that dilutes the black to blue (grey) and the yellow to beige interacting with the basic colours influenced by the agouti and extension mutations (Castle 1930, Robinson 1958, Searle 1968). In other species, similar phenotypic effects are determined by mutations in a few genes including the premelanosome protein (*PMEL*) gene, also known as melanocyte protein Pmel 17 (*PMEL17*) or silver (*SILV*). PMEL plays a central role in the biogenesis of the early stages of the pigment organelle, the melanosomes (Theos *et al.* 2005). In stage II melanosomes, processed PMEL protein aggregates to form fibrils, to which presumably the eumelanin is attached (Berson *et al.* 2001). Mutations within the *PMEL* gene altering the function of the coded protein are associated with the diluted-like of the basic coat colour in mouse, cattle, horse, dog and chicken (Martinez-Esparza *et al.* 1999, Kerje *et al.* 2004, Oulmouden *et al.* 2005, Brunberg *et al.* 2006, Clark *et al.* 2006, Gutiérrez-Gil *et al.* 2007, Kühn & Weikard 2007).

To evaluate the potential role of the rabbit *PMEL* gene in determining the *dilute* locus, we isolated and sequenced *PMEL* cDNAs as well as fragments of intronic and exonic regions and identified single nucleotide polymorphisms (SNPs), one of which was used to evaluate linkage with the dilute mutation in domestic rabbits.

Materials and methods

Animals

Twelve rabbits with different coat colours were used for resequencing of parts of the *PMEL* gene. This group of animals was made by: one Belgian Hare rabbit; 4 Vienna Blue rabbits, expected to be homozygous for the mutated dilute allele; 7 Checkered Giant rabbits, one with blue spots (buck), expected to be homozygous for the mutated dilute allele; 6 with black spots, 2 of which (does) expected to be heterozygous for the mutated dilute allele (as

they were obtained by crossing a black spotted buck with 2 other blue spotted does that were not possible to sample) and other 4 putative homozygous animals for the wild type allele at this locus, as deduced by pedigree information. In addition, two half sib-families with 5 and 7 F1 were produced crossing the sampled blue Checkered Giant buck with the two heterozygous (but black) Checkered Giant does. The full-length *PMEL* cDNA (see below) was obtained from dorsal skin of a Rex rabbit exhibiting wild type phenotype (Fontanesi *et al.* 2010a). Two additional rabbits of different breeds (Angora and Chinchilla) were used for *PMEL* cDNA isolation from dorsal skin as described below.

DNA/RNA extraction and cDNA synthesis

Genomic DNA was extracted from hair roots as previously described (Fontanesi *et al.* 2007) or from blood (collected after slaughtering of the animals in a commercial abattoir) using a standard protocol. Dorsal skin specimens were collected from three rabbits after slaughtering, immediately frozen in liquid nitrogen and then stored at −80 °C prior to RNA isolation that was carried out as already reported (Girardot *et al.* 2005). Reverse Transcription was performed from l µg of DNase treated total RNA in a volume of 20 µL, using 0.5 µg oligo (dT) primer and SuperScript II RNase H^- Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The reaction was incubated for 50 min at 42 °C and then for 15 min at 70 °C.

Rapid Amplification of cDNA Ends (RACE) and full-length cDNA cloning

To isolate the rabbit full-length *PMEL* cDNA, we performed RACE experiments with 5 µg of total RNA extracted from dorsal skin of a Rex rabbit using the SMART RACE cDNA Amplification kit (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France), according to the manufacturer's instructions. Consensus primers (Table 1) were designed based on coding sequence homology between human and bovine *PMEL* gene (GenBank acc. no. NM_006928 and Oulmouden *et al.* 2005). Nested PCR with consensus and adaptor primers was used for 5'-untranslated regions (UTR) and 3'-UTR amplification as previously described (Fontanesi *et al.* 2010a, Table 1). PCR products were cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA, USA) and sequenced as described below. The 5'- and 3'-end sequences were determined and used to design specific primers (sense and anti-sense) to amplify full-length cDNAs from the Rex rabbit and two additional animals, one Angora and one Chinchilla (Table 1).

Identification and analysis of polymorphisms in rabbit families

Four PCR primer pairs were designed on the rabbit *PMEL* gene sequence (Ensembl no. ENSOCUG00000000999) to amplify the coding regions of 4 exons and parts of downstream or upstream intronic sequences (Table 1 and Figure 1). Genomic DNA used for PCR amplification and sequencing was from the 12 rabbits already described above. PCR was carried out using a PTC-100 thermal cycler (MJ Research, Watertown, MA, USA) in a 20 µL reaction volume containing ~50 ng genomic DNA, 1 U DNA EuroTaq DNA polymerase (EuroClone Ltd., Paington, Devon, UK), 1X Euro Taq PCR buffer, 2.5 mM dNTPs, 10 pmol of each primer and 2.0 mM of $MgCl_2$. PCR profile was as follows: 5 min at 95 °C; 35 amplification cycles of 30 sec at 95 °C, 30 s at 56-62 °C, 30 s at 72 °C; 5 min at 72 °C (Table 1). The amplified fragments

Table 1
PCR primer pairs and PCR conditions

Primer name and amplified region	Primer forward and *reverse* sequences (5'-3')	PCR[2]	Use
5'-UTR first primer	CAATGGGAGCCAGGTGTGGGGAGGAC	-	5'-RACE
5'-UTR second primer	AATGATGGGCCTACACTGATTGGKGC	-	5'-RACE
5'-UTR first primer	YMCCCAGCCCAGCCTGCCAGCTGGTT	-	3'-RACE
5'-UTR second primer	GTCAGCACCCAGCTTRTCATGCCTGG	-	3'-RACE
Specific cDNA	GGCCTTTGGTTGCTGGAGAGAAGAAC *CTTCCTTCAGAGGCAGCCATGACCCG*	2091/61/1.5	Full length CDS amplification of *PMEL* cDNA
Exon 1 region[1]	TGTAGCAGGGTGGGGAAG *CCAAACCAAATACATGGGAGA*	399/59/2.0	Re-sequencing genomic region
Exon 2 region[1]	CCATGGGAGACCAGGAGAA *AGACTCAAAGCGGCACTCAG*	396/55/1.0	Re-sequencing genomic region
Exon 5 region[1]	GGCCCCAGACTTCCGAAT *GCTTCTCCTAGCCCTGTGAC*	354/58/2.0	Re-sequencing genomic region
Exon 7 region[1]	GCTCTGGGTCTACCTGGAAA *GAGGTGCAAGGTTAAAGACTGG*	446/59/2.0	Re-sequencing genomic region, PCR-RFLP with *Ssi*I

[1]Including intronic regions as described in *Results and discussion*. [2]Information is related to the different primer pairs: amplified product size (bp)/annealing temperature (°C)/[$MgCl_2$] (mM). For the primers used in the RACE experiments, only the specific *PMEL* primers are reported (PCR conditions are the same as described in Fontanesi *et al.* [2010a]).

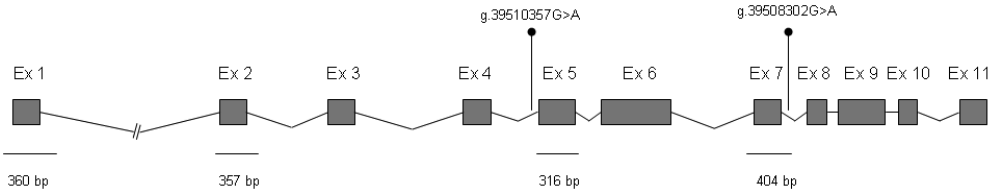

Figure 1
Structure of the rabbit *PMEL* gene (Ensembl no. ENSOCUG00000000999), re-sequenced regions from genomic DNA (indicated below the schematic representation of the gene), and position of the two intronic SNPs. Size of the re-sequenced regions does not include primers.

were sequenced after treatment with 1 µL of ExoSAP-IT® (USB Corporation, Cleveland, Ohio, USA) for 15 min at 37 °C. Cycle sequencing of the treated PCR products was produced using the same PCR primers and the Big Dye v3.1 kit (Applied Biosystems, Foster City, CA, USA). Sequencing reactions, after a few purification steps using EDTA, Ethanol 100 % and Ethanol 70 %, were loaded on an ABI3100 Avant capillary sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were edited and aligned with the help of the CodonCode Aligner software (CodonCode Corporation, Dedham, MA, USA).

PCR-RFLP (Table 1) was applied to genotype the g.39510357G>A SNP (nomenclature of intronic SNPs is based on system coordinates of rabbit chromosome 4 in the oryCun2.0

genome version: http://www.ensembl.org/Oryctolagus_cuniculus/Info/) in the rabbit F1 families. Briefly, the amplified fragment of 446 bp obtained using the same exon 7 primers used for re-sequencing was digested with *Ssi*I. Digestion reaction was carried out overnight at 37 °C in a 25 µL reaction volume including 5 µL of PCR product, 1× restriction enzyme buffer and 2 U of *Ssi*I (MBI Fermentas, Vilnius, Lithuania). The resulting DNA fragments were electrophoresed on 10 % 29:1 polyacrylamide:bis acrylamide gel and visualized with 1× GelRed Nucleic Acid Gel Stain (Biotium Inc., Hayward, CA, USA). Restriction patterns were as follow: two fragments (353+93 bp) when allele A was present; one fragment of 446 bp if allele G was present. The PCR product obtained from 3 F1 rabbits was also sequenced as described before in order to confirm the PCR-RFLP genotyping results.

Results and discussion

Rabbit PMEL cDNA sequences

The rabbit *PMEL* cDNA sequence obtained from the Rex rabbit included 29 bp of the 5'-UTR, 1 974 bp of the coding sequence (CDS) and 131 of the 3'-UTR (with the polyA tail), for a total of 2 135 bp (GenBank/EMBL acc. no.: FR849710). The coding sequence encoded for a predicted 657 amino acid sequence, that was 79 %, 82 %, 80 % and 78 % identical to the human (661 amino acids, P40967), horse (662 amino acids, NP_001157361), pig (636 amino acids, BAE03309) and cattle (649 amino acids, Q06154) PMEL amino acid sequences, respectively. As expected, the predicted rabbit PMEL amino acid sequence has the characteristic signature for mammalian PMEL protein (Theos *et al.* 2005). A short N-terminal signal sequence of 24 residues is followed by a N-terminal region (NTR) of 200 amino acids that contains three putative N-terminal glycosylation sites and three cysteine residues. Downstream of the NTR is a polycystic kidney disease (PKD) domain followed by a conserved cysteine residue that separates a region rich in acidic residues, prolines, serines and threonines consisting primarily of imperfect direct repeated sequences (repeat domain, RPT). Following the RPT region is a conserved dibasic sequence that serves as a site for proteolytic cleavage that is critical for PMEL function. Downstream of the cleavage site is a conserved region known as the Kringle-like domain (KLD), characterized by the presence of highly conserved cysteines. Following a highly conserved cysteine and N-glycosylation site at the C-terminus of the KLD is a poorly conserved, short proline-rich region, the length of which varies among the two major splice forms in humans that separates the KLD from the single 24-residue membrane spanning domain.

Aligning the Rex rabbit cDNA sequence with that obtained from Angora and Chinchilla rabbits (GenBank/EMBL acc. no.: FR849711 and FR849709) and the rabbit cDNA sequence XM_002711060 (retrieved from GenBank and derived by automated computational analysis with gene prediction methods of sequencing data of the rabbit genome version oryCun2.0, that, in the meantime, has been completed at the level of ~7X by the Broad Institute) we identified, on the whole, 10 SNPs, 8 of which are missense polymorphisms (c.557T>G, p.V186G; c.584A>G, p.Q195R; c.603T>C; c.854C>T, p.P285L; c.878C>A, p.P293Q; c.1016A>T, p.Q339L; c.1256C>T, p.T419M; c.1346G>T, p.S449I; c.1501G>A, p.G501R; c.1770T>C), and 2 are synonymous SNPs (c.603T>C; c.1770T>C).

Re-sequencing of parts of the rabbit PMEL gene and identification of intronic polymorphisms

To identify polymorphisms that could be useful to evaluate their possible co-segregation with the black-blue spotted phenotype in the F1 families, fragments of the rabbit *PMEL* gene including 4 out of 11 exons were re-sequenced in rabbits with different putative alleles at the dilute locus (Figure 1). These fragments encompassed exon 1 and upstream flanking and downstream intronic regions (360 bp), exon 2 with part of preceding intron 1 and downstream intron 2 regions (357 bp), exon 5 and parts of intron 4 and parts of intron 5 (316 bp), exon 7 and portions of upstream and downstream introns 6 and 7 (404 bp). Within a total of 1 437 bp re-sequenced fragments, only 2 single nucleotide polymorphisms were identified in intronic regions: one was in intron 4 (g.39510357G>A); the second was in intron 7 (g.39508302G>A). Sequences have been submitted to GenBank/EMBL with acc. no. FR849712 and FR849713. These SNPs are not in any apparently functional or regulatory motif.

Exclusion of the rabbit PMEL gene as the determinant of the dilute locus

The two identified SNPs were in linkage disequilibrium in the sequenced rabbits. Therefore we selected one of them (g.39510357G>A) for the analysis of the rabbit families in which there was segregation of blue and black coat colours. The genotyping of the animals was carried out by PCR-RFLP and sequencing of some of the analysed samples confirmed the genotyping results. Figure 2 shows pedigree information including the genotypes and the coat colour of the animals. The results clearly indicated that there is no co-segregation between the g.39510357G>A SNP and the coat colour of the F1 rabbits. Black and blue rabbits share the same genotypes. Therefore, the *PMEL* gene can be excluded as a possible determinant of the dilute coat colour locus in rabbit. It remains to be investigated if polymorphisms in this gene can have other effects on pigmentation, like the silvering in Champagne d'Argent breed, or can modify the intensity of the blue colour in rabbits showing this phenotype. Other identified cDNA polymorphisms will be analysed to this purpose.

We have already excluded another strong candidate gene [myosin VA (heavy chain 12, myoxin), *MYO5A*] for the dilute locus in rabbits (Fontanesi *et al.* 2012) that in mouse is responsible for the corresponding dilute locus (Mercer *et al.* 1991). Therefore, the exclusion of the *PMEL* gene further shorten the list of other possible candidate genes that have been already shown to determine hypopigmentation or dilution of coat colour in other species, like melanophilin (*MLPH*), or RAB27A, member RAS oncogene family (RAB27A) (Wilson *et al.* 2000, Matesic *et al.* 2001).

The characterization of this locus could add basic information to the biology of pigmentation in mammals as well as potential applications for fancy breeders, that mainly in countries like Germany, France, Italy and USA, contribute to maintain and develop rabbit breeds and lines.

Acknowledgements

This work was funded by University of Bologna RFO and FaGenomicH projects and by INRA/ University of Limoges, and was carried out in the framework of the COST Action TD1101 »A Collaboratve European Network on Rabbit Genome Biology – RGB-Net«.

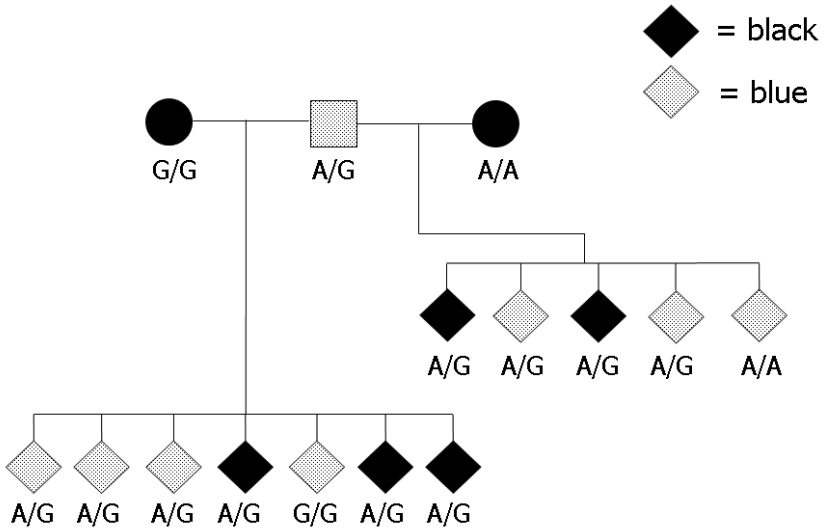

Figure 2
Genotype of the g.39510357G>A SNP of animals of the F1 families in which there is segregation of the black-blue spotted coat colours.

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