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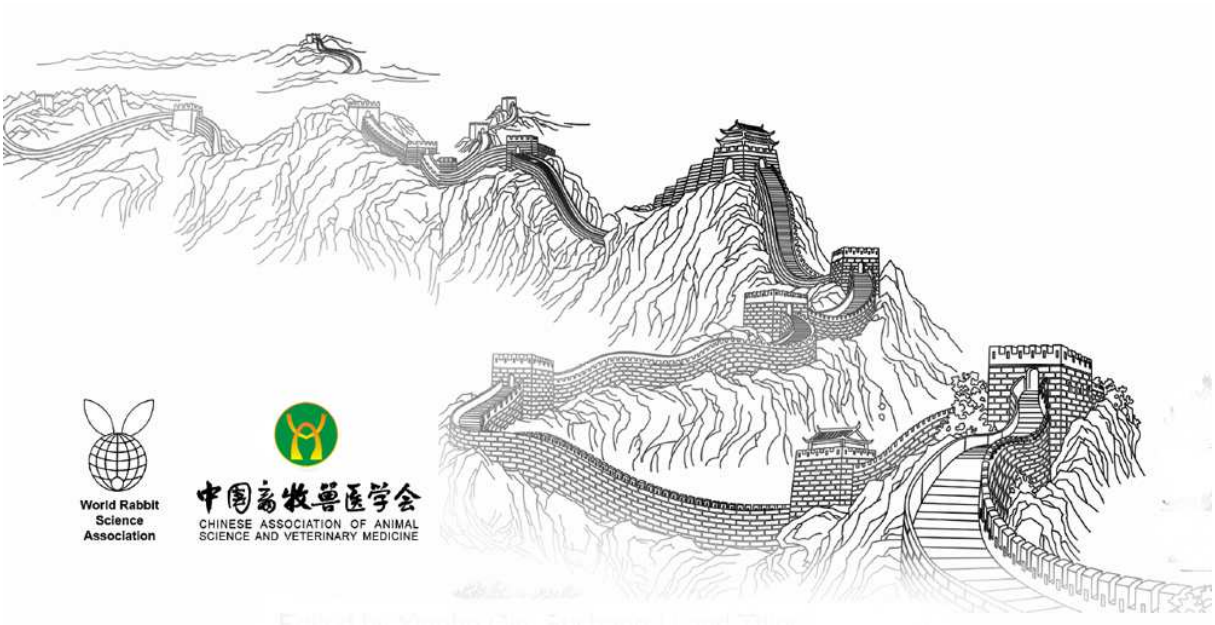

THE *MLPH* EXPRESSION IS DECREASED IN RABBITS OWNING A DILUTION OF COAT COLOUR

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ABSTRACT

Coat colour dilution is a specific pigmentation phenotype which segregates in many mammalian species. This trait affects both eumelanin and pheomelanin synthesis and corresponds to a dilution of wild type pigments. In human, mutations within genes encoding for the proteins of the tripartite complex (as example the melanophilin, MLPH), cause Griscelli syndromes characterized by a dilution of the hair colour. In rabbit, the "*Dilute*" locus is determined by a major gene with an autosomal recessive genetic determinism. Gene candidate approaches focused on the *MLPH* gene highlighted 2 distinct polymorphisms associated with the "Dilute" phenotype in rabbits. Both mutations located either at a splice acceptor site or into the exon 6 of the gene might affect the stability of *MLPH* messengers. To evaluate the effect of one of the two mutations, we quantified the *MLPH* transcripts levels in skin samples of animals owning a dilution of coat colour or a wild type phenotype. Castor and Chinchilla breeds in which the interest phenotype exists were considered; unrelated 16 "Dilute" rabbits (8 of each strain) and 16 wild type animals (8 of each breed) were selected. First of all, we genotyped the whole dataset for the 1bp non-synonymous deletion. All "Dilute" rabbits are homozygous $MLPH^{d/d}$ while wild type individuals are homozygous for the reference allele $MLPH^{wt/wt}$. Our results confirmed this polymorphism as the potential causal mutation of the coat colour dilution. Then, the *MLPH* expression was measured to reinforce the involvement of this gene and this specific mutation into the "Dilute" phenotype. In both breeds, a drastic and significant decreased of the *MLPH* transcripts levels was shown in rabbits $MLPH^{d/d}$ and owning a "Dilute" phenotype (p-values ranging from 10^{-03} to 10^{-06}). Altogether, our data strongly support the importance of the deletion identified within the MLPH gene and brought new insights into the coat colour dilution trait.

Key words: coat colour, dilution, melanophilin, deletion, Castor and Chinchilla breeds

INTRODUCTION

Different coat colours in the European rabbit (*Oryctolagus cuniculus*) have been selected through the domestication process and then fixed in particular strains. Among the various phenotypic traits highlighted, a particular pattern of pigmentation corresponding to a dilution of the coat colour is known as the "Dilute" phenotype. A human disorder, the Griscelli syndromes, causes the dilution of the hair accompanied by further symptoms. Mutations within the *melanophilin* gene (*MLPH*) have been described in some affected patients (Sanal et al., 2002).

A candidate gene approach focused on the *MLPH* gene has been performed in a population of rabbit representative of various breeds for which some of them owning a dilution of their coat (Fontanesi et al., 2014). The rabbit dilution allele (d) is a monogenic autosomal recessive trait (Pap, 1921). A 1bp deletion into the exon 6 of the *MLPH* gene has been identified as a putative causal mutation responsible of the "Dilute" phenotype (Fontanesi et al., 2014). Another polymorphism, a single nucleotide polymorphism, located within an intron and affecting a splice acceptor site within the polypyrimidine tract has also been associated with the coat colour dilution in rabbits (Lehner et al., 2013).

To support the potential role of the highlighted deletion in the dilution of the coat colour, we investigated its impact on the stability of MLPH messengers in skin of "Wild Type" or "Dilute" rabbits.

MATERIALS AND METHODS

Animals and experimental design

The "Dilute" phenotype has been observed within the Castor and Chinchilla breeds (Figure 1). The mutation identified by Fontanesi et al. (2014) segregated in both strains. To perform our study, 16 rabbits owning a wild type phenotype (8 Castor and 8 Chinchilla) and 16 animals carrying a dilution of their coat colour (8 Castor and 8 Chinchilla) have been selected. Unrelated individuals have been chosen. Skin samples have been collected for all rabbits at 3 months age.

Figure 1: Characterization of the "Dilute" phenotype in Castor and Chinchilla strains. The rabbits owning a wild type coat colour are shown in (a) and (c) for the Castor and Chinchilla breeds respectively while pictures in (b) and (d) represent the Castor and Chinchilla "Dilute" animals respectively.

Chemical Analyses

DNA and RNA extractions. Genomic DNA and total RNA were extracted from the same skin biopsy using the Qiagen AllPrep DNA/RNA Mini kit. 20µl cDNA was obtained from 1µg total mRNA using Invitrogen Life Technologies Supercript II Reverse Transcriptase First Strand cDNA Synthesis and random primer.

Genotyping of the "Dilute" deletion. Specific allele PCR was used to genotype the deletion. The three primers used (Table 1) were designed on the rabbit *MLPH* sequence (ENSOCUG00000016496) with the Primer3 software. PCR was carried out using a 2720 thermal cycler (Life Technologies) in a 12µl reaction volume containing 20ng genomic DNA, 0.5U GoTaq DNA polymerase (Promega), 1X GoTaq PCR buffer, 0.2mM dNTPs, 0.4µM of each primer and 1.5mM MgCl2. PCR profile was as follow: 5min at 95°C, 35 amplification cycles of 30s at 95°C, 30s at 66°C for the deleted allele or 68°C for the wild type allele, 30s at 72°C, 10min at 72°C. The PCR products were electrophoresed on 2% agarose gel and visualized with bromide of ethidium.

Table 1: List of primers used for both the genotyping and the expression analyses

Couple	Primer1	Primer2	PCR fragment size	Comment
Wild Type allele	ATCCACGACCTGGACTTGG	ACTCTGCACCCTGGTCTGAA	179	genotyping
Deleted allele	ATCCACGACCTGGACTTGA	ACTCTGCACCCTGGTCTGAA	179	genotyping
HPRT(1)	GGCAAAACAATGCAGACCTT	CTTCGAGGGGTCCTTTTCAC	95	qPCR
MLPH_ex4_ex5	ATGGGCTCTCTCGAGTGGTA	GGTCGCTGTCTCCACTTCTC	148	qPCR
MLPH_ex5_ex6	GCCTTGAGGAGAGAAGTGGA	AGGTCGTGGATGGAGAGGAG	118	qPCR
MLPH_ex13_ex15	TACAGAGCCTCCTGGTGAAG	ACTTCCGGTCAAGAGAGACC	107	qPCR
KAP1(2)	TCCAGACGTTCTAGGCTGAC	TCCCTACTCAAGTGCAGAGC	114	qPCR

[1]HPRT for Hypoxanthine-guanine PhosphoRibosylTransferase and [2]KAP for Keratin Associated Protein 1.

mRNA stability and accumulation *via* quantitative PCR. qPCR analysis was carried out with a LightCycler® 480 System using the SYBR Green I Master (Roche Life Science) as recommended by the manufacturer. Briefly, 3µL of diluted cDNA (1/5) was mixed with 0.15µM of each primer and 1X Master volume in a final volume of 10µL and a classical qPCR amplification cycle was then performed as follow: 45 cycles with 15s at 95°C, 15s at 60°C and 15s at 72°C. Each qPCR point was done in duplicate.

Statistical Analysis

Quantitative PCR data were estimated according to Pfaffl et al. (2001) as relative expression versus the *HPRT* housekeeping gene. For each genotypic group containing 8 samples, the mean of relative expression and standard deviation (SD) were calculated. The significance of results was tested using a t-test for the comparison of the genotypic clades.

RESULTS AND DISCUSSION

A first step was to genotype the whole dataset for the deletion identified by Fontanesi et al. (2014). All Castor and Chinchilla rabbits owning a dilution of their coat colour ("Dilute" animals, n=16) were homozygous for the (d) allele while all wild type individuals (n=16) were homozygous for the (wt) allele (data not shown). Another polymorphism into the *MLPH* gene, affecting a splice acceptor site and resulting to exons-skipping, was associated with the coat colour dilution (Lehner et al., 2013). Unfortunately, this SNP was not genotyped in our individuals. We may partially conclude that our results reinforced the role of the 1bp non-synonymous deletion as the potential causal mutation of the "Dilute" phenotype but we can't exclude the implication of the mutation identified by Lehner et al. (2013).

To evaluate the consequence of the 1bp deletion on the *MLPH* expression, we studied the accumulation and stability of messengers in both wild type and "Dilute" rabbits. We firstly determined the best housekeeping gene to choose for further analyses. A total of 10 genes were tested according to those suggested by Vuckovic et al. (2013) and we obtained the least variability and the best reproducibility with the *HPRT* gene (data not shown). We then kept working with this housekeeping gene which was the most stable in skin samples. Our results were different from those observed in whole foetal lung tissue since *HPRT* presented a very low expression (Vuckovic et al., 2013).

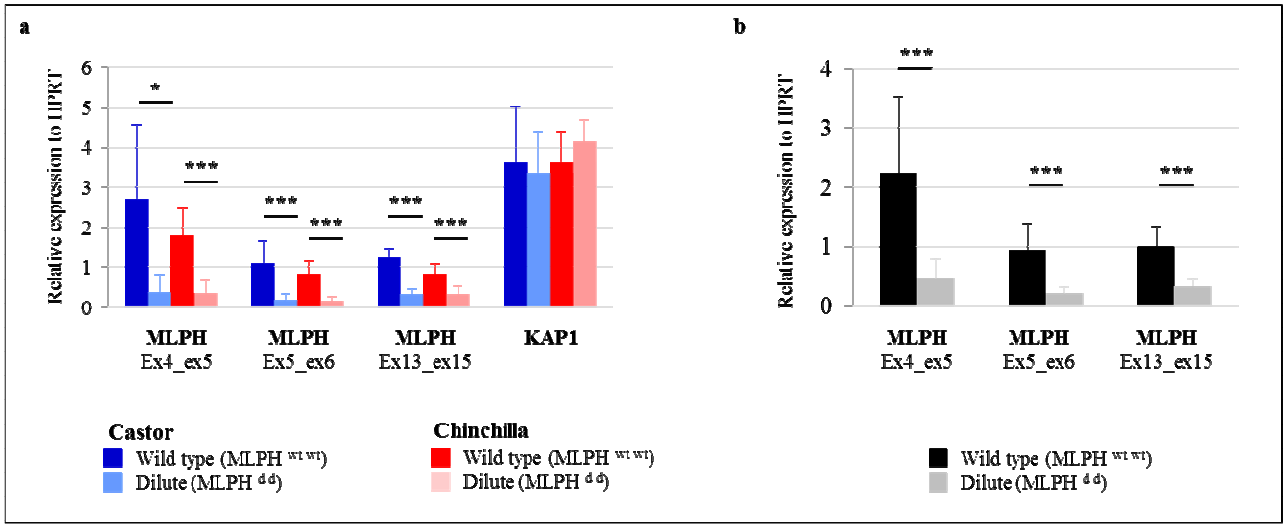

Figure 2: Quantification of the *MLPH* expression in skin samples of rabbits owning either a wilt type or a "Dilute" phenotype. **(a)** Comparison of data intra-breeds (Castor in dark and light blue and Chinchilla in dark and light red). **(b)** Comparison of date inter-breeds (wild type in black vs. "Dilute" in grey).

Several pairs of primers designed into the MLPH mRNA were tested to make a valid conclusion concerning the expression of the gene in the skin biopsies of "Dilute" animals (Table 1). In addition, we choose the *KAP1* gene which is a Keratin Associated Protein as a positive control of the experiment because KAP1 is well known to be highly expressed in keratinocytes (Wu et al., 2008).

Similar results were observed for the various couples of primers and in both Castor and Chinchilla breeds with a significant decrease of the MLPH expression in rabbits with a coat colour dilution (Figure 2a). The comparison of phenotype without considering the origin of strain was even more powerful and also showed a significant difference of messengers between the wild type $MLPH^{wt/wt}$ group of animals and the "Dilute" clade carrying the $MLPH^{d/d}$ genotype (Figure 2b). It is important to mention that despite the

very low level of expression of the *MLPH* gene in "Dilute" rabbits leading to a very high variability of data representing by standard deviation bars, the differences observed seemed highly significant ($p<10^{-05}$ as showed on Figure 2b). Our results are in concordance with those obtained for others species (cats and dogs) (Ishida et al. 2006, Drogemuller et al. 2007). Indeed, mutations located into the *MLPH* gene and associated with a dilute phenotype were predict to alter the splicing and/or the protein. In dogs, quantitative PCR showed that dilute animals had only about 25% of the *MLPH* transcript compared with wild type animals (Drogemuller et al., 2007).

CONCLUSIONS

The dilution of coat colour in rabbits gained some insights thanks to our study which showed that *(i)* the *HPRT* gene seemed the pertinent housekeeping gene for skin tissue, *(ii)* the 1bp deletion into the exon 6 of the *MLPH* gene might be the causal mutation of the "Dilute" phenotype in Castor and Chinchilla breeds and *(iii)* the level of MLPH messengers was drastically decreased in animals carrying the $MLPH^{d/d}$ genotype and owning a diluted coat colour.

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