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Letter to the editor

A 2-bp insertion (c.67_68insCC) in *MC1R* causes recessive white coat color in Bama miniature pigs

Coat color is an important characteristic of various breeds of domestic animal species. Variation in farm animal coat color is of considerable interest for concealment, communication and protection against solar radiation (Slominski et al., 2004). It also plays an important role in the regulation of physiological processes (Miyagi and Terai, 2013). Bama miniature pigs are a native breed originally from southern China with a "Liang-tou-wu" coat color pattern (black coat on the head and bottom and white coat on the body, also called two-end black). Bama miniature pigs have been widely used as experimental animals in biomedical research because of their clear genetic background, high fertility, light body weight, and mostly white coat color. Many genetically modified Bama miniature pigs have been established as human disease models, such as the wound and burn healing (Sun et al., 2008), lipid metabolism diseases (Zhang et al., 2010), cardiovascular diseases (Zhang et al., 2014), blood diseases (Hai et al., 2014), neurodegenerative diseases (Wang et al., 2016), and auditory system diseases (Zhou et al., 2016). In addition, the "Liang-tou-wu" coat color is the unique coat color pattern among indigenous Chinese pig breeds, but the molecular basis remains elusive. Understanding the molecular mechanism responsible for pig coat color can provide important genetic information for the breeding of white experimental animals and the study of melanoma (Schook et al., 2015).

In the current study, we identified a Bama mutant pig with a white coat color pattern with only very small black areas (Fig. 1A). To obtain this mutant line, a wild-type boar (1200501) was mated with a sow (018T031) that gave birth to 19 offspring in 2 litters. Six of the nineteen offspring exhibited a white coat color with only very small black spots, which fit the Mendelian segregation ratio of 1:3 (6:13, χ^2 test, $P > 0.05$) (Fig. 1B and C), suggesting that this mutation may be a Mendelian recessive inheritance pattern. The recessive inheritance of the phenotype was further confirmed by crossing two white mutant pigs, the offspring of which all presented white coat color (Fig. 1B). The H&E staining results showed that the hair shaft of the mutant skin lacked pigmentation (Fig. 1D). Since the hair shaft consists of elongated cells containing melanin in the cytoplasm and the melanin content determines the color of the hair, we speculated that the causative mutation might be related to the synthesis and migration of skin melanin.

KIT proto-oncogene receptor tyrosine kinase (*KIT*) plays an important role in the survival and migration of neural-crest-derived melanocyte precursors. It has been reported that the *Dominant white* allele (*I*) of *KIT* carrying a 450-kb duplication mutation and a splice site mutation at the first nucleotide of intron 17 causes the white coat color (Andersson, 2009), which is quite similar to our mutant phenotype. Therefore, we first genotyped the *Dominant white* allele (*I*) of *KIT* in this Bama mutant line. The 152-bp fragment spanning the *KIT* duplication breakpoint was not observed in Bama pigs (Fig. S1) (Giuffra et al., 2002). The sequencing results also showed that the splice site mutation in *KIT* intron 17 was not found in Bama pigs (data not shown). These data suggest that the *Dominant white* allele (*I*) of *KIT* is not responsible for the mutant phenotype in Bama miniature pigs.

A genome-wide linkage study (GWLS) was subsequently employed to map the causative mutation. Ten F_1 pigs (including six mutant and four two-end black pigs) and their parents were genotyped using Porcine SNP60 Chips (Illumina, USA) (Fig. 1B). The GWLS revealed a strong linkage signal in the chr6: 0–10 Mb region (LOD = 3.5092) (Fig. 1E and Table S1). There are 143 coding genes in this region, 30 of which have been annotated (Table S2). Among those annotated genes, melanocortin receptor 1 (*MC1R*) is the only pigmentation-related gene that is expressed in melanocytes. In addition, this gene is known to play an important role in melanogenesis by controlling the production of red/yellow pheomelanin and dark eumelanin (Barsh, 1996). Previous studies have demonstrated that *MC1R* variants are associated with different coat colors in numerous animal species, including mice (Robbins et al., 1993), dogs (Newton et al., 2000), cattle (Klungland et al., 1995), chickens (Takeuchi et al., 1996), pigs (Kijas et al., 1998) and others. Thus, *MC1R* was regarded as a candidate gene responsible for the mutant phenotype in this mutant line.

To screen the mutations in *MC1R*, the coding sequence and partial 5′-UTR and 3′-UTR were amplified and sequenced (Fig. S2 and Table S3). A 2-bp CC insertion mutation (c.67_68insCC) was identified and co-segregated with the mutant phenotype completely (Fig. 1F and Table S4). This CC insertion at codon 23 results in a frameshift mutation (p.Arg23ProfsTer33) that produces a prematurely terminated, nonfunctional receptor (Fig. 1F). To date, seven *MC1R* alleles have been reported, which result in four different coat color phenotypes: wild type (*MC1R*-E*$^+$, *MC1R*1-E*$^+$), dominant black (*MC1R*2-E*^{D1}, *MC1R*7-E*^{D1} and *MC1R*3-E*^{D2}), black spotting (*MC1R*6-E*^{P}) and recessive red (*MC1R*4-e*) (Andersson, 2003). The underlying genetic characteristic of the E^P allele is this CC insertion mutation, which makes it possible to distinguish E^P from other alleles, such as E^+, E^{D1}, E^{D2} and *e* (Kijas et al., 1998). In this study, mutant pigs were genotyped as homozygous E^P/E^P. Furthermore, this CC insertion in *MC1R* was also found in Yorkshire and Landrace pigs using direct sequencing (Tables 1 and S4). A previous report has shown

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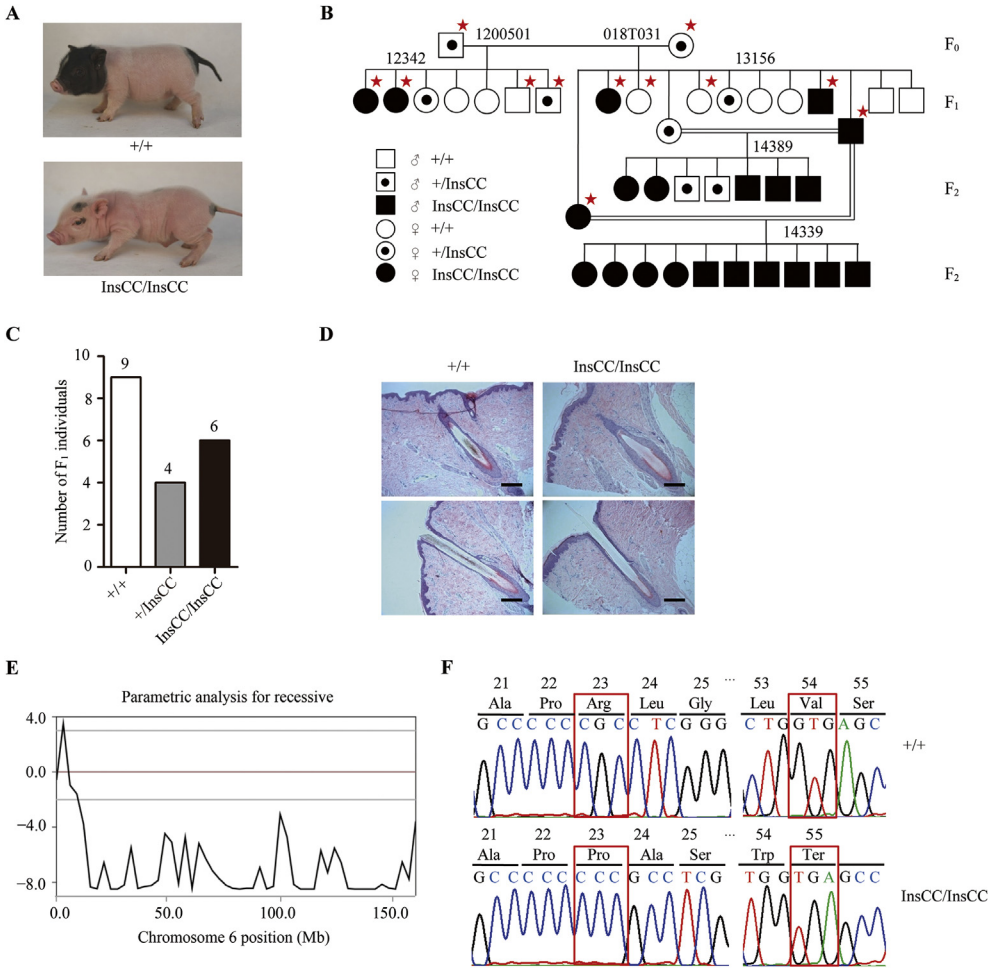

Fig. 1. The frameshift resulting from the 2-bp CC insertion (c.67_68insCC) in *MC1R* generates a white coat color phenotype in Bama miniature pigs. **A:** Phenotypic characteristics of wild-type (+/+) and mutant (InsCC/InsCC) Bama miniature pigs. The upper image shows the wild-type phenotype, which is "Liang-tou-wu", and the lower image shows the mutant phenotype, which is white with small black spots. **B:** Representative pedigree of the mutant line. The individuals used for whole genome SNP genotyping are indicated with red stars on the top right corner. **C:** Genotype statistics for the pigs in F_1 generation shown in **B**. **D:** H&E staining of the skin from wild-type and mutant Bama miniature pigs. The white hair shaft of mutant Bama pigs showed no melanin. Bars = 200 μm. **E:** Genome-wide linkage study revealed a strong linkage signal on chr6: 0–10 Mb region (LOD = 3.5092). **F:** The 2-bp CC insertion (c.67_68insCC) results in a frameshift mutation (p.Arg23ProfsTer33) and premature termination of translation.

that most E^P/E^P homozygotes are red with black spots or white with black spots, with a few appearing uniformly red (Andersson, 2009), while the Yorkshire and Landrace pigs showed a completely white coat color without any black spots because of the epistatic effects of the *Dominant white* allele (*I*) of *KIT* (Marklund et al., 1998).

To investigate the effects of the *MC1R* CC insertion mutation on the expression of other related coat color genes, we performed qRT-PCR for *MC1R*, *MITF* (microphthalmia-associated transcription factor), *TYR* (tyrosinase), *TYRP1* (tyrosinase related protein 1) and *DCT* (dopachrome tautomerase) using cDNA from the head skin of two-end black and mutant Bama pigs. The results showed that the expression of *MITF*, *TYR*, *TYRP1* and *DCT* was significantly decreased in mutant pigs relative to two-end black pigs, whereas the expression of *MC1R* was not significantly altered, suggesting that this mutation did not mediate a decay in *MC1R* mRNA (Fig. S3). In addition, we analyzed the growth data and found that only the body weight on day 0, day 15 and day 60 but not at other measurement points were significantly different between two-end black and mutant pigs (Table S5). We also measured the clinical blood physiological and biochemical parameters. For blood physiology, 18 parameters were examined, and significant differences were observed in HGB (hemoglobin), RDW (red cell distribution width), MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin) and MPV (mean platelet volume) between two-end black and mutant pigs

Table 1
Identification of the 2-bp CC insertion (c.67_68insCC) in *MC1R* with direct sequencing.

Breed	Coat color	Number	Genotype			Allele frequency (%)	
			+/+	+/InsCC	InsCC/InsCC	+	InsCC
Bama	White with black spots	12	0	0	12	0	100
	Two-end black	32	16	16	0	75	25
Yorkshire	White	30	0	0	30	0	100
Landrace	White	29	0	0	29	0	100
Duroc	Red	35	35	0	0	100	0

(Table S6); for blood biochemistry, 15 parameters were detected, and significant differences were observed in BUN (blood urea nitrogen), CRE (creatinine), GLU (glucose) and LDL (low-density lipoprotein cholesterol) levels between two-end black and mutant pigs (Table S7). The significant differences in the blood physiological and biochemical parameters between the two-end black and mutant pigs provided us with a direction to further investigate the impact of the *MC1R* mutation on pig metabolism. Previous studies have shown that *MC1R* and *MITF* confer a genetic predisposition to melanoma (Eggermont et al., 2014). The pig is an ideal system for studying cancer since porcine cells are resistant to transformation in a manner similar to human cells (Schook et al., 2015). Therefore, the changes in coat color caused by the *MC1R* mutation have the potential to provide preclinical models for studying melanoma.

In summary, we identified a 2-bp CC insertion (c.67_68insCC) in the *MC1R* gene in Bama miniature pigs that results in a white coat color phenotype inherited in a recessive pattern. This mutant Bama miniature line provides an important large animal model for medical research.

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Supplementary data

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