Biallelic \( \beta \)-carotene oxygenase 2 knockout results in yellow fat in sheep via CRISPR/Cas9

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Summary

The recently emerged CRISPR/Cas9 approach represents an efficient and versatile genome editing tool for producing genetically modified animals. \( \beta \)-carotene oxygenase 2 (BCO2) is a key enzyme in the progress of \( \beta \)-carotene metabolism and is associated with yellow adipose tissue color in sheep. We have recently demonstrated targeted multiplex mutagenesis in sheep and have generated a group of BCO2-disrupted sheep by zygote injection of the CRISPR/Cas9 components. Here, we show that biallelic modification of BCO2 resulted in yellow fat, compared with the fat color in monoallelic individuals and wild types (snow-flower white). We subsequently characterized the effects of gene modifications at genetic levels employing sequencing and Western blotting, highlighting the importance of the BCO2 gene for the determination of fat color in sheep. These results indicate that genetic modification via CRISPR/Cas9 holds great potential for validating gene functions as well as for generating desirable phenotypes for economically important traits in livestock.

Keywords genetic engineering, genome editing, \textit{Ovis aries}, phenotype, \( \beta \)-carotene oxygenase 2

The metabolism of \( \beta \)-carotene is a nutritionally important pathway to provide vitamin A, which is essential for mammals. \( \beta \)-carotene is involved in a number of beneficial functions in mammals due to its ability to generate vitamin A \textit{in vivo} as well as to take part in crucial signaling functions of its metabolites. Moreover, carotene also works as an antioxidant (Shete & Quadro 2013). In the course of carotene metabolism, two key enzymes—\( \beta \)-carotene 15,15\textsuperscript{0}-monooxygenase 1 (BCMO1) and \( \beta \)-carotene oxygenase 2 (BCO2)—play an important role. BCO2 can cleave the \( \beta \)-carotene asymmetrically to generate a \( \beta \)-ionone ring and apocarotenals, which can be translated into retinaldehyd (Von Lintig 2010, 2012). The yellow adipose tissue phenotype is closely related to the accumulation of carotenoids (Crane & Clare 1975; Kirton \textit{et al.} 2012). Evidence has shown that mutations in BCO2 are associated with carotenoid metabolism in adipose tissues. The nonsense mutation c.196C>T in the BCO2 gene is associated with yellow fat in Norwegian sheep (Vage & Boman 2010). Similar results were found in cows; mutations in BCO2 were associated with the accumulation of \( \beta \)-carotene in milk and serum (Berry \textit{et al.} 2009) as well as in fat tissues (Tian \textit{et al.} 2010). In rabbits, the yellow fat resulted from a biallelic AAT deletion in the BCO2 gene (Strychalski \textit{et al.} 2015). ‘Yellow fat’ is regarded as a simple recessive trait in sheep (Baker \textit{et al.} 1985); thus, we set out to disrupt BCO2 through zygote injection of sgRNAs and Cas9 mRNA mixtures in an attempt to evaluate the color of adipose tissues of gene-modified animals.

By employing the CRISPR/Cas9 system, we successfully generated gene-modified Tan sheep (Wang \textit{et al.} 2016). Cas9 mRNA and two sgRNAs targeting exon 2 of the BCO2 gene were microinjected intracytoplasmically into one cell stage sheep zygotes (Fig. 1a, Table S1), which were collected surgically from naturally mated sheep as previously described by Wang \textit{et al.} (2016). We were able to obtain a total of 10 Tan sheep with the BCO2 gene disrupted. To investigate whether BCO2 disruption leads to fat color changes in sheep, we here report the consequences of gene modification at the genetic, expression, and protein levels.

According to the previous sequencing results based on TA clones using whole blood samples (Wang \textit{et al.} 2016), we
defined founder animals without any wild type (WT) genotype as biallelic modified animals and founder animals with a monoallelic WT genotype as monoallelic modified animals. We next collected adipose tissues from the tails of 12-month-old sheep with different genotypes, which were subjected to T7E1 cleavage and Sanger sequencing. The genomic DNA was extracted using the Universal Genomic DNA Kit (CWBIO, CW2298M) and amplified with Primer-STAR HS DNA polymerase (TaKaRa, DR010A). The PCR products were purified with E.Z.N.A Cycle-Pure Kit (Omega, D6492-02) and separated by electrophoresis on a 2–2.5% agarose gel (Invitrogen, 75510-019). Genotyping was conducted using T7EI (NEB, M0302L) cleavage assay and TA clone sequencing. The T7EI cleavage assay showed that the adipose tissues of modified sheep contained various edited genotypes (Fig. 1d), and further TA sequencing confirmed that the T7EI assay results (Table 1, Fig. S1), and the genotypes [biallelic knockout (KO), monoallelic knockout and wild type] in the adipose tissues were consistent with the genotyping results using blood samples (Wang et al. 2016).

To determine the fat colors, fresh adipose tissues from tails of three biallelic \( \text{BCO2} \)-KO sheep, three monoallelic \( \text{BCO2} \)-KO sheep, and three wild types were sampled and immediately loaded onto a cardboard with a white background. Interestingly, the adipose tissues from all biallelic \( \text{BCO2} \)-KO sheep exhibited a yellow color compared...
with the snow-flower white fat in monoallelic and WT individuals (Fig. 1b). In industrial color criteria, every pixel is represented by the three parameters of red (R), green (G) and blue (B), which indicate the mixture of these three primary colors to achieve the pixel’s color. We further determined the yellow color by calculating the (R+G)/B values, which were obtained through Adobe Photoshop (10.0.1) software (Table S2), and the biallelic animals displayed a significant shift to yellow fat color compared to the other two groups (Fig. 1c).

We next sought to determine whether the BCO2 expression was inhibited at the transcriptional or translational levels. Because we were unable to conduct quantitative PCR to evaluate mRNA expression for each genotype in a given mosaic founder, we amplified cDNA fragments to determine whether the modified genotypes yielded clear gel bands. Total RNA was extracted from adipose tissues using a MiniBEST Universal RNA Extraction Kit (TaKaRa, 9767) and the inverse transcript was determined with a PrimeScript™ RT Reagent Kit (TaKaRa, RR037A). We next amplified the cDNA with a RT-PCR primer (Table S3), and variable bands were found among the biallelic, monoallelic and WT animals (Fig. 1e). Surprisingly, as shown in Fig. 1e, fewer bands were found in Bi-1 and Bi-2 compared with the PCR products using genomic DNA (Fig. 1d), indicating the intensity of targeted alleles was decreased. TA sequencing of cDNA products further verified the absence of transcripts in Bi-1, Bi-2 and other founder animals (Table 1, Fig. S1). We assume that the mosaic animals carrying more than two alleles were induced by non-homologous end joining repair, whereas some of the alleles may not have been transcribed during the transcription process.

To further verify whether knocking out the BCO2 gene with different genotypes would influence expression at the translational level, Western blotting was subsequently implemented to confirm the lack of protein expression in nine animals. Using RIPA Lysis Buffer (Biotime, P0013E), we extracted total protein from adipose tissues which were separated by SDS/PAGE and transferred onto a PVDF membrane (Roche, 3010040001). The polyclonal antibodies were selected specifically for BCO2 (1:1000, abcam, ab199297). As expected, three biallelic individuals showed the absence of expression of BCO2 at the protein level (Fig. 1f), whereas the protein expression in the monoallelic and WT animals was not affected. The result indicates that BCO2 lost its function at the protein level only in the biallelic modified sheep.

In summary, our findings demonstrate that the phenotypes of biallelic BCO2 knockout animals were significantly altered in comparison with monoallelic and WT animals. We further highlight the merits of sgRNA:Cas9-mediated genome editing as a powerful approach to generate animals with desirable phenotypes as well as for the functional validation of key genes underlying important traits in livestock.

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References


Supporting information

Additional supporting information may be found online in the supporting information tab for this article:

Figure S1 Sequencing results of modified BCO2 gene locus and mRNA detected in adipose tissue.

Table S1 sgRNA sequences and target sites.

Table S2 R, B, G values of adipose tissue color.

Table S3 Primers used for amplification of the BCO2 gene segment and its cDNA.