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REGULAR ARTICLE

Expression of *OPA1* and *Mic60* genes and their association with mitochondrial cristae morphology in Tibetan sheep

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Abstract

In order to investigate the relationship between the expression of *OPA1* and *Mic60* genes and the shape of mitochondrial cristae and to explore the mechanism of Tibetan sheep adapting to a high altitude hypoxia environment, we investigate respiratory rate, mitochondrial cristae and the expression of *OPA1* and *Mic60* in four different tissues (myocardial, skeletal muscle, spleen and kidney) in Tibetan sheep and Small Tail Han sheep. Tibetan sheep had a higher respiratory rate than Small Tail Han sheep ($p < 0.01$). In the same tissue, the expression of *OPA1* and *Mic60* was higher ($p < 0.05$) in Tibetan sheep than Small Tail Han sheep. Between tissues, the expression of *OPA1* and *Mic60* was found to be lower ($p < 0.05$) in spleen than the other three tissues in both breeds. Mitochondrial cristae was dense and clear in myocardial and skeletal muscle but was relatively sparse and slightly swollen in kidney. In spleen, cristae was least and swollen and the gap between the cristae was large. The width of the mitochondrial cristae in the spleen was significantly larger than the width between the inner and outer membranes; however, it had little difference in the other three tissues. The width of mitochondrial cristae was significantly larger in the spleen than that in other tissues ($p < 0.05$). The numbers of mitochondrial cristae in the four tissues of Tibetan sheep were larger than those in Small Tail Han sheep ($p < 0.05$). The unique characters of the mitochondrial cristae in Tibetan sheep may be related to its adaption to a high altitude hypoxia environment.

Keywords *OPA1* gene · *Mic60* gene · Mitochondria · Cristae · Tibetan sheep

Introduction

Tibetan sheep are mainly distributed in the Qinghai-Tibet Plateau and its adjacent areas at the altitude of 3000~5000 m above sea level, which is one of the main economic sources of local farmers and herdsmen. By the end of 2008, there were over 23 million Tibetan sheep in China (Du 2011). Mitochondria, as the "energy factory" in eukaryotic cells, is the main place for aerobic respiration and plays an important role in maintaining intracellular calcium homeostasis (Dimmer and Scorrano 2006) and regulating the apoptosis process (Frezza et al. 2006). The integrity of mitochondrial morphology and structure, especially the structural integrity of the cristae, directly affects the function of mitochondria (Cogliati et al. 2013).

The mitochondrial cristae membrane is the main place of oxidative phosphorylation, where many proteins and enzymes associated with oxidative phosphorylation are located (Chaban et al. 2014; Vogel et al. 2006). More than 90% of the mitochondrial oxidative phosphorylation complex III and ATP synthase are found in the mitochondrial cristae membrane (Gilkerson et al. 2003).

Optic atrophy 1 (OPA1) is mainly located in the mitochondrial membrane space (Carelli et al. 2007). MICOS complex subunit Mic60 is a transmembrane protein on the membrane of the mitochondria located in the intermembrane space (IMS) (Gieffers et al. 1997). OPA1 and Mic60 play an important role in the maintenance of mitochondrial cristae morphology. Some studies have shown that the nuclear gene *OPA1* is

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expressed in various tissues and organs but the expression levels vary in different tissues or organs (Olichon et al. 2007). OPA1 can promote the fusion of mitochondria (Youle and van der Bliek 2012) and the defect of the *OPA1* gene can cause the fragmentation of the mitochondria and destroy the fusion of mitochondrial network (Spinazzi et al. 2008). When the OPA1 protein is inactivated or its synthesis is inhibited, the intracellular mitochondrial tubular network structure will break up (Meeusen et al. 2006). The deletion of the OPA1 subtype leads to the malformation of the mitochondrial cristae morphology and has a serious influence on the proliferation of the cells (Griparic et al. 2004; Griparic et al. 2004).

The mitochondrial inner membrane organizing system (MINOS) is composed of six subunits: Fcj1/Mic60 (Mitofilin), MINOS1/Mio10, Mcs12 (Aim5), Mcs19 (Aim13), Mcs27 (Aim37) and Mcs29 (Mos2) (Alkhaja et al. 2012; Harner et al. 2011; Hoppins et al. 2011). As the core component of the MINOS complex, Mic60 is particularly important for the maintenance of mitochondrial cristae morphology (Von der Malsburg et al. 2011). Interference with the expression of Mic60 not only will result in an abnormal mitochondrial structure but also will obviously inhibit the normal function of mitochondria. Knockdown of the *Mic60* gene has been reported to interfere with mitochondrial transcription and oxidative-phosphorylation activity (Yang et al. 2015). The transmembrane domain of Mic60 protein is mainly responsible for anchoring proteins to the inner membrane of mitochondria. The spiral domain and carboxyl terminal affect the formation of the mitochondrial cristae junction (Koerner et al. 2012). It has been reported that the inhibition of Mic60 expression in HeLa cells by siRNA treatment led to an increase in cell apoptosis and a decrease in proliferation level and in these cells, the mitochondrial cristae membrane was transformed from a normal tube into onions and the number of cristae junctions was significantly reduced (John et al. 2005). By interacting with a variety of mitochondrial proteins, the MINOS complex participates in the maintenance of mitochondrial cristae morphology and regulates the morphology of the mitochondrial cristae membrane. Researchers found that MOMA-1, CHCH-3 and Mic60 were involved in the maintenance of mitochondrial cristae membrane morphology in *Caenorhabditis elegans* using RNA interference screening technology (Head et al. 2011).

At present, studies on the adaptability of Tibetan sheep to hypoxia at high altitude mostly focus on molecular regulation mechanism, physiology and anatomy and little has been carried out on the specificity of the mitochondrial ultrastructure. In this study, we combine the molecular regulation mechanism and the observation and comparative study of the mitochondria ultrastructure to further reveal the mechanism of adaptation to the high altitude hypoxia environment of Tibetan sheep.

Materials and methods

Materials

Three ewes of Tibetan sheep from Gannan Luqu (altitude of approximately 3500 m) and three ewes of Small Tail Han sheep from Lanzhou City (altitude 1500–2000 m) were slaughtered according to the conventional method. All ewes were approximately 3 years old. Samples of myocardial, skeletal muscle, spleen and kidney tissues were collected immediately after slaughter and kept on ice during the process of cutting. One part of the tissue samples was cut 1 mm × 1 mm × 2 mm, cleaned with PBS, fixed in 3% glutaraldehyde solution and then stored at 4 °C for transmission electron microscopy analyses. The other part of the tissue samples was rapidly frozen in liquid nitrogen and then stored in the refrigerator at − 80 °C for *OPA1* and *Mic60* gene expression.

Respiratory rate

Respiratory rate was measured in 11 ewes of Tibetan sheep and 11 ewes of Small Tail Han sheep. All ewes were approximately 3 years old and healthy. The total number of ups and downs of the chest was counted within a minute at rest.

Extraction of tissue RNA

The RNA was extracted from tissue samples using the RNA Simple Total kit, following the manufacturer's instructions. The quality of RNA extracted was tested by 1.5% agarose gel electrophoresis and the concentration was measured by an ultra-micro spectrophotometer. The RNA samples were stored at − 80 °C.

Design and synthesis of PCR primers

The GenBank sequences of sheep *OPA1* and *Mic60* genes (accession number: XM_012140446 and XM_012169573) were used to design PCR primers. The β-actin (NM 001009784) was used as the reference gene. Primers were synthesized by BGI Liuhe Company (Beijing). Detailed information of primers is shown in Table 1.

Reverse transcription-quantitative PCR

The total RNA used for RT-qPCR was synthesized into cDNA using a FastKing RT kit (with gDNase). The resulting cDNA was diluted ten times and then used for qPCR analysis. qPCR was performed in triplicate using the relative quantitative SYBR Green I dye method in 20-μL reaction containing 2 μL diluted cDNA template (< 100 ng), 0.4 μL each of 0.25 μM PCR primer, 10 μL of 2 × ChamQ SYBR qPCR Master Mix (Vazyme), 0.4 μL ROX II and 6.8 μL of

Table 1 Primer information for sheep *OPA1*, *Mic60* and β-actin genes

Gene	Primer sequence (5′-3′)	Primer length (bp)	Product Length (bp)	Annealing temperature (°C)
OPA1	F:ATGAAATAGAACTCCGAATG R:GTCAACAAGCACCATCCT	20 18	112	60
Mic60	F:TTGAGATGGTCCTTGGTT R:TTGTTTCTGAGGTGGTGAG	18 19	136	60
β-actin	F: AGCCTTCCTTCCTGGGCATGGA R:GGACAGCACCGTGTTGGCGTAGA	22 23	113	60

RNase-free ddH_2O. The conditions of qPCR reaction were as follows: an initial denaturation at 94 °C, 5 min; 40 cycles of denatured at 94 °C, 15 s, annealing at 60 °C, 34 s, extension at 72 °C, 30 s; 40 cycles. The dissolution reaction contained denaturation 15 s at 94 °C and fluorescence signal 60 s at 60 °C. The data were collected and then the gene expression was calculated (Livak and Schmittgen 2001). The variance of gene expression was analyzed by SPSS software.

Electron microscopy analysis

The cut sections of tissues were fixed with 1% osmic acid at 4 °C for 2 h and then rinsed twice (10 min each) with 0.2 mM PB solution. After dehydration in graded ethanol (50% ethanol for 10 min, 70% ethanol for 10 min, 80% ethanol for 10 min, 90% ethanol for 10 min), the cut sections were dehydrated in 100% acetone twice (10 min for each time). The sections were soaked in the mixture of Epoxy resin and acetone (1:1) for 5 h, then embedded in epoxy resin and dried at 45 °C for 12 h, followed by 65 °C for 48 h. The samples were sectioned. Semi-thin sections were used for location, while the ultrathin sections were stained with uranium citrate and lead citrate and the ultrastructure of mitochondrion was observed by a JEM-1230 transmission electron microscope (Japan).

Results

Respiratory rate

The respiratory rate of Tibetan sheep was 49.72 ± 7.01 times/min, which was significantly higher ($p < 0.01$) than that of Small Tail Han sheep (44.18 ± 4.31 times/min).

Analysis of the result of RT-qPCR

Qualitative analysis of the expression of *OPA1* gene and *Mic60* gene of Tibetan sheep and Small Tail Han sheep in the myocardium, skeletal muscle, kidney and spleen tissues was analyzed by the method of RT-qPCR. The results showed that both *OPA1* and *Mic60* genes were expressed in all of these four tissues (Fig. 1a).

The Tm value of the two gene amplification products was uniform and the melt curve showed a single peak, indicating that both primer sets had good specificity in the RT-qPCR reaction and that non-specific amplification and primer dimers were not observed (Fig. 1b).

Among Tibetan sheep and Small Tail Han sheep, the expression of *OPA1* gene was the highest in the myocardium followed by skeletal muscle and kidney and was the lowest in the spleen. In Tibetan sheep, the expression levels of *OPA1* gene in skeletal muscle and kidney were 0.561 ± 0.03 and 0.425 ± 0.035 times of that in myocardium, and the expression level in spleen was 0.114 ± 0.015 times of that in the myocardium. In Small Tail Han sheep, the expression of *OPA1* gene in myocardium was 0.228 ± 0.055 times of that in Tibetan sheep. The expression of skeletal muscle and kidney was 0.150 ± 0.026 and 0.140 ± 0.020 times of that in Tibetan sheep's myocardium. The expression in the spleen was 0.046 ± 0.020 times of that in Tibetan sheep's myocardium

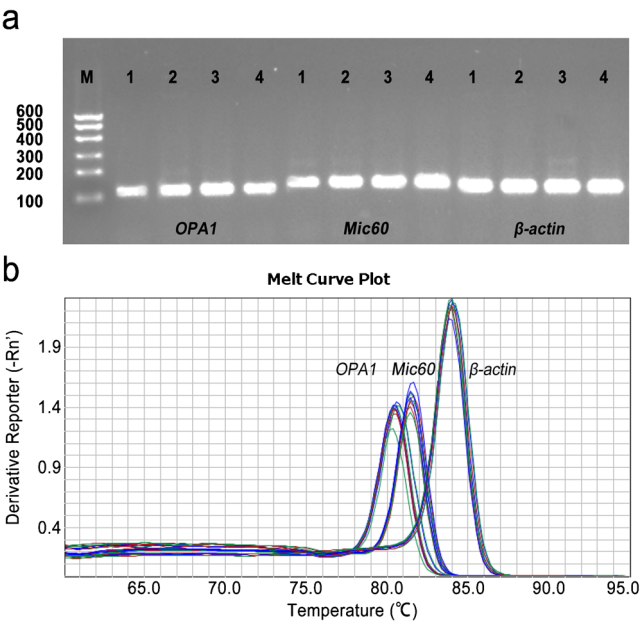

Fig. 1 RT-PCR electrophoresis pattern (**a**) and melting curves (**b**) of *OPA1* gene, *Mic60* gene and β-actin in four tissues of ewes. Note: 1 skeletal muscle, 2 myocardium, 3 spleen, 4 kidney

(Fig. 2a). Among Tibetan sheep and Small Tail Han sheep, the expression of *Mic60* gene was the highest in the skeletal muscle, followed by the myocardium and kidney and was the lowest in the spleen. In Tibetan sheep, the expression of *Mic60* gene in skeletal muscle and kidney was, respectively, 1.196 ± 0.020 and 1.036 ± 0.081 times of that in myocardium. The expression in spleen was 0.378 ± 0.040 times that in myocardium. For Small Tail Han sheep, the expression of *Mic60* gene in myocardium was 0.92 6 ± 0.036 times that in Tibetan sheep's myocardium. The expression of skeletal muscle and kidney was, respectively, 1.047 ± 0.041 and 0.752 ± 0.056 times of that in Tibetan sheep's myocardium. The expression in spleen was 0.138 ± 0.078 times of that in Tibetan sheep's myocardium (Fig. 2b). The expression levels of *OPA1* gene and *Mic60* gene in myocardium, spleen, skeletal muscle and kidney of Small Tail Han sheep were significantly lower than those of Tibetan sheep ($p < 0.05$) (Fig. 2a, b).

The comparison of mitochondria

Both Tibetan sheep and Small Tail Han sheep had a clear double membrane and tube cristae in all of the four tissues but the cristae of mitochondria were denser in Tibetan sheep than in Small Tail Han sheep. Mitochondria in myocardial and skeletal muscle had clear membranes and clear and dense cristae. Mitochondria in kidney had clear membranes but the cristae were relatively sparse and slightly swollen. Mitochondrial cristae in the spleen were least and swollen, with a large gap between cristae (Fig. 3).

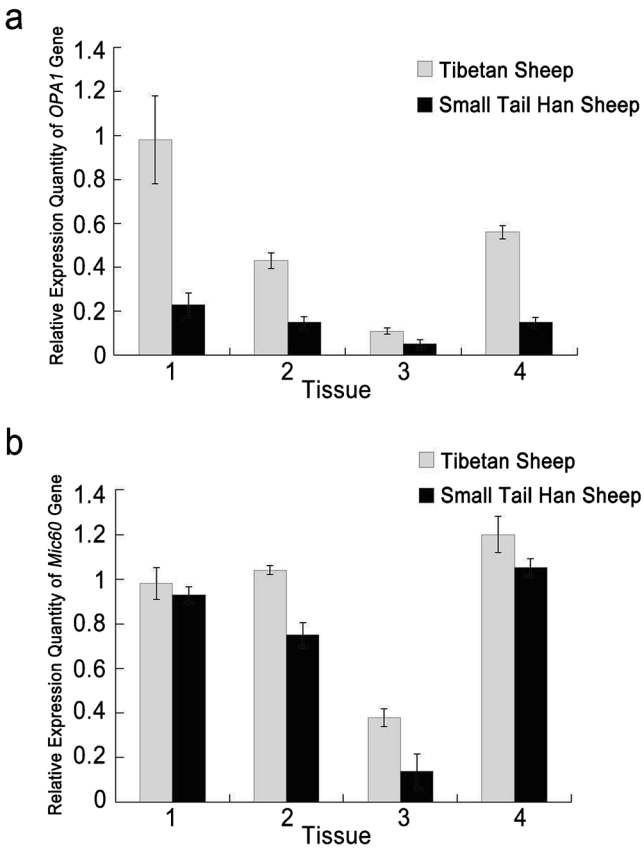

Fig. 2 Expression of *OPA1* gene (**a**) and *Mic60* gene (**b**) in different tissues of Tibetan Sheep and Small Tail Han Sheep. Note: 1 myocardium, 2 kidney, 3 spleen, 4 skeletal muscle. Tibetan Sheep myocardium represents control tissue; error bars indicate standard deviation

Mitochondria in heart, kidney and skeletal muscle had little difference between cristae width (19.75 ± 1.08 nm, 17.72 ± 1.62 nm, 11.65 ± 1.42 nm, respectively, in Tibetan sheep; 20.86 ± 2.26 nm, 20.76 ± 0.97 nm, 17.16 ± 1.72 nm, respectively, in Small Tail Han sheep) and the outer-inner membrane width (20.25 ± 2.77 nm, 15.77 ± 1.38 nm, 10.15 ± 0.93 nm, respectively, in Tibetan sheep; 23.62 ± 2.81 nm, 14.23 ± 1.08 nm, 16.08 ± 1.91 nm, respectively, in Small Tail Han sheep). However, the width of the mitochondrial cristae in the spleen (27.58 ± 1.51 nm in Tibetan sheep; 29.94 ± 1.30 nm in Small Tail Han sheep) was significantly larger than the width between the inner and outer membranes (12.46 ± 1.68 nm in Tibetan sheep; 12.99 ± 1.74 nm in Small Tail Han sheep) and significantly larger than that in other tissues ($p < 0.01$) (Fig. 4d).

The numbers of mitochondrial cristae in myocardium, kidney, spleen and muscle of Tibetan sheep were 31.2 ± 1.99, 16.2 ± 1.63, 7.8 ± 1.11 and 16.8 ± 1.83, respectively. In Small Tail Han sheep, the numbers of mitochondrial cristae in myocardium, kidney, spleen and muscle were 16.6 ± 1.35, 8.2 ± 2.15, 6.8 ± 1.07, and 10 ± 2.91, respectively. The average number of mitochondrial cristae in the four tissues was larger in Tibetan sheep than in Small Tail Han sheep ($p < 0.05$) (Fig. 4e).

Discussion

This study found that the expression of *OPA1* gene in the tissue was positively correlated with the number of mitochondrial cristae, that is, the mitochondria in the tissue with high OPA1 expression had more cristae. A recent study found that with the decrease in OPA1 protein, a large number of cristae in mitochondria disappeared in siRNA transfected Hela cells (Griparic et al. 2004). This is consistent with the results of our study. We suspect that the reason may be that the decrease of OPA1 causes the inhibition of mitochondrial fusion. Patten et al. (2014) reported that OPA1 played a regulatory role in mitochondrial cristae that was independent of its fusion function; therefore, the decrease of cristae may also be the result of OPA1 acting directly on the mitochondrial cristae. The specific mechanism needs to be further studied.

It was also found that the expression of *Mic60* gene was negatively correlated with the width of mitochondrial cristae, that is, the cristae of mitochondria was wider in the tissues with lower expression of *Mic60*. Von der Malsburg et al.

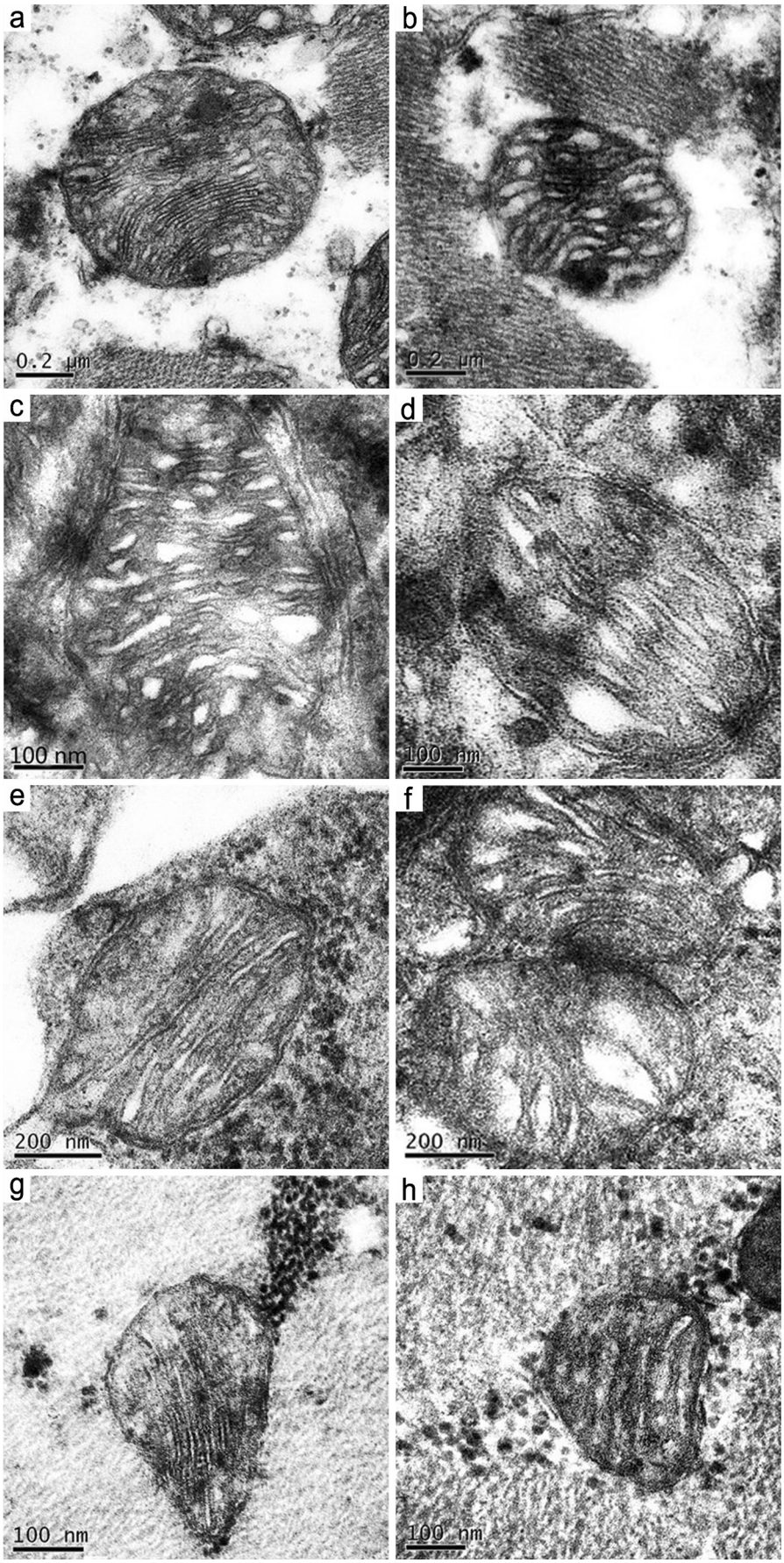

Fig. 3 Representative electron micrographs of mitochondria in different tissues of Tibetan sheep (**a**, **c**, **e**, **g**) and Small Tail Han sheep (**b**, **d**, **f**, **h**). Note: **a**, **b** myocardium, bar: 0.2 μm. **c**, **d** Kidney, bar: 100 nm. **e**, **f** Spleen, bar: 200 nm. **g**, **h** Skeletal muscle, bar: 100 nm

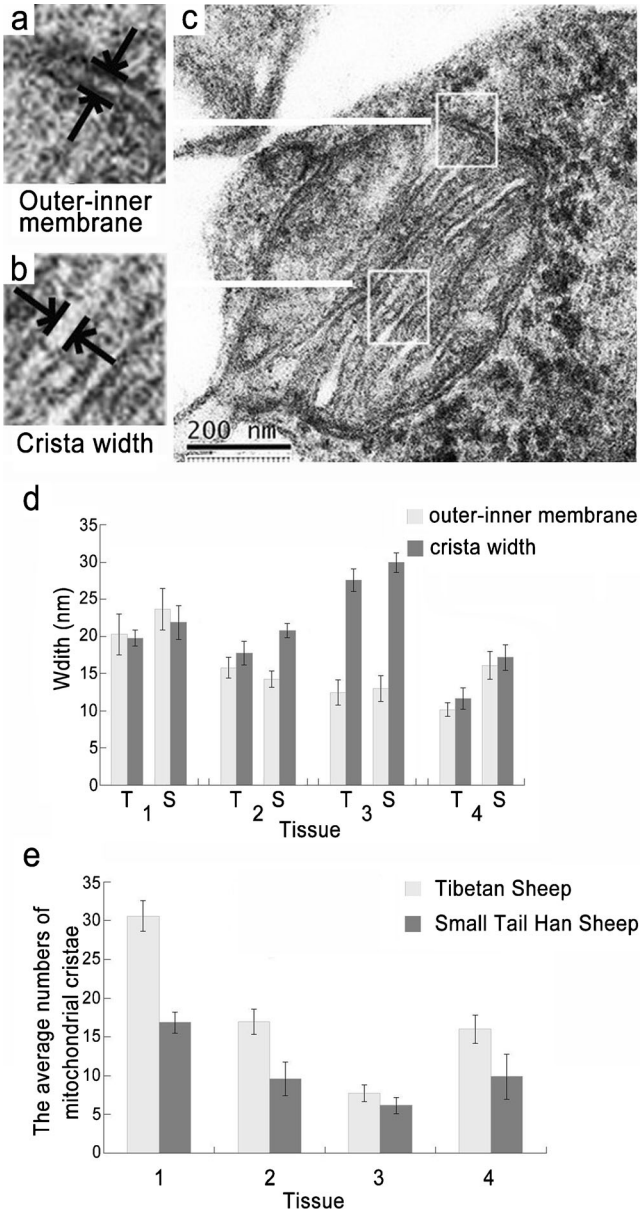

Fig. 4 The width of outer-inner membrane and crista (**d**) and the quantity of mitochondrial crista in myocardium, kidney, spleen and skeletal muscle (**e**). Note: T Tibetan Sheep. S Small Tail Hank Sheep. 1 Myocardium, 2 kidney, 3 spleen, 4 skeletal muscle. Error bars indicate standard deviation

(2011) found that in the fcj1-deficient yeast cells, the morphology of mitochondrial cristae changed significantly, the cristae membrane became hypertrophic and stacked, the cristae junctions were lost and the cristae and the inner boundary membrane were separated. Patten et al. (2014) found that there were more oligomeric OPA1 in the starved cells, while the width of the cristae in the starved cells was obviously narrow, suggesting that OPA1 plays a role in the regulation of the cristae width. The results from this study suggest that both OPA1 and Mic60 play a role in regulating the width of the cristae. OPA1 and Mic60 may interact with each other in the process of regulating cristae morphology (Darshi et al. 2011) but the specific mechanisms are not known yet. Barrera et al. (2016) found that in the cells of OPA1$^{-/-}$, the amount of Mic60 increased to combat apoptosis. Mic60 is essential for the formation of cristae junctions. While OPA1 plays an important role in the regulation of crest morphology, the formation of cristae junctions is not necessary (Barrera et al. 2016).

Mitochondrial cristae increase the mitochondrial inner membrane surface area and what is more, they provide attachment sites for the oxidative phosphorylation-related protein and enzyme. The number of mitochondria varies in different cells; it is usually considered that the more mitochondrial cristae, the higher oxidative phosphorylation ability and also the higher cell metabolic activity and energy consumption.

Compared to the expression of *OPA1* gene and *Mic60* gene in the same tissue, the expression level was significantly higher in Tibetan sheep than in Small Tail Han sheep ($p < 0.05$). Mitochondria in Tibetan sheep had more and denser cristae, suggesting that the oxidative phosphorylation ability of mitochondria in Tibetan sheep is higher and has a better utilization of oxygen. Given that the respiratory rate was significantly higher in Tibetan sheep than in Small Tail Han sheep, Tibetan sheep may be able to breathe more oxygen in a certain period of time. This is probably the result of the adaptation of Tibetan sheep to the low oxygen environment in the plateau. The expression of *OPA1* and *Mic60* genes in spleen was significantly lower than that in myocardium, skeletal muscle and kidney ($p < 0.05$) and the number of mitochondrial cristae in spleen was significantly less than that in other tissues ($p < 0.05$). This may be because the spleen is relatively inactive among these four tissues.

Conclusion

The expression of *OPA1* and *Mic60* genes had an organizational difference and a variety of differences. Within breeds, the expression of *OPA1* and *Mic60* genes was highest in myocardium, followed by skeletal muscle and kidney and was the lowest in the spleen. Between breeds, the expression of *OPA1* and *Mic60* genes was higher in Tibetan sheep than in Small Tail Han sheep.

The expression of *OPA1* and *Mic60* genes was associated with the number and morphology of mitochondrial cristae. The mitochondria in myocardium, spleen, kidney and skeletal muscle had clear double membranes and tubular cristae and a complete structure. The number of mitochondrial cristae in the myocardium, spleen, kidney and skeletal muscle of Tibetan sheep was more than that in the corresponding tissues of Small Tail Han sheep. These results suggest that the number and morphological specificity of the mitochondrial cristae in Tibetan sheep are the ultrastructural basis of the cells adapted to the high altitude hypoxia environment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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