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Comparative analysis of tissue expression and methylation reveal the crucial hypoxia genes in hypoxia resistant animals

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ABSTRACT

Tibetan goat and Tibetan sheep are species peculiar to Qinghai-Tibetan Plateau which is the highest plateau in the world, and have high hypoxia resistance to the extremely bad environment. Unlike monogastrics, how the genes change responding to hypoxia in ruminants remains unclear. In the present study, three healthy animals of every breeds, including Tibetan sheep, Tibetan goat, Chuanzhong goat and Small-Tailed Han sheep were selected. The expression of factor inhibiting HIF 1 (*FIH-1*), hypoxia inducible factor 1 α (*HIF-1 α*), *HIF-3 α* and erythropoietin (*EPO*) in various tissues, including heart, liver, lungs, kidney, muscle and brain, were investigated. *EPO* was observed highly expressed in all the tissues of Tibetan goats and Tibetan sheep compared with low-altitude animals respectively, implied that higher expression of *EPO* may give the explanation for the hypoxia resistance of plateau animals. Besides, we also cloned the promoters of *FIH-1*, *HIF-1 α* , *HIF-3 α* and *EPO* in goats and sheep, analyzed their core regions and CpG islands. Higher methylation rate was observed in *HIF-1 α* , *HIF-3 α* and *EPO*, while lower methylation rate hit on *FIH-1*. These data may be beneficial

for revealing the response mechanism to hypoxia of plateau animals.

Key Words: Qinghai-Tibetan Plateau, goat, sheep, tissue expression, hypoxia resistant

Short Title: Crucial hypoxia genes in hypoxia resistant animals

INTRODUCTION

The Qinghai-Tibetan plateau is the highest plateau at an average altitude of 4000 meters above sea level, and is known as “the roof of the world”. The cold climate and hypoxia are the two most important ecological factors restricting plateau animals. Response to O₂ deprivation of organisms is a complex biological and physiological process (Zhao et al. 2004). The native plateau animals must have developed their own mechanisms in adapting to extremely low oxygen concentration during their long evolutionary history.

The most two significant hypoxia defense mechanisms exist in the animal society are severe down-regulation of energy turnover and up-regulation of the energetic efficiency of ATP-producing pathways (Hochachka 1986; Lutz and Storey 1997). Hypoxia inducible factors (*HIFs*), contain *HIF-1*, *HIF-2* and *HIF-3* (Maynard et al. 2007), as crucial regulators for maintaining biological homeostasis under low oxygen (hypoxic) stress, are mostly non-functional in oxygenated cells. Under low oxygen stress, it becomes active and promotes or represses the transcription of a broad range of genes by binding to the hypoxia response elements (*HRE*) in their promoters, to involve in hypoxia resistance regulation (Brahimi-Horn and Pouyssegur 2009). *HIF* is a heterodimeric complex composed of an oxygen-destructible α -subunit and an oxygen-indestructible β -subunit. Three isoforms of the α -subunit are involved in the response to hypoxia in vivo (Brahimi-Horn and Pouyssegur 2009). The regulation of *HIFs* are mainly at the level of protein stability and transcriptional activity in an oxygen-dependent manner by prolyl and asparaginyl hydroxylation (Peet and Linke 2006). The inhibition of post-transcriptional hydroxylation of the α -subunits were required for *HIF*

transcription activation (Brahimi-Horn and Pouyssegur 2009). Factor inhibiting HIF (*FIH-1*) is the only known asparaginyl hydroxylase of *HIF*, and targets a conserved asparaginyl residue within the C-terminal activation domain (*CAD*) of *HIF- α* (Peet and Linke 2006). Hence, it is well-believed that the mRNA expression and the post-translational modification of *HIF- α* and *FIH* may play important roles for the hypoxia resistance of native plateau animals.

Based on the importance of *HIF-1 α* in hypoxia resistance, it was hypothesized that hypoxia genes may change their expression and methylation to adapt to the extremely low oxygen environment. In the present study, we checked the tissues expression divergence of *FIH-1*, *HIF-1 α* , *HIF-3 α* and erythropoietin (*EPO*), a main target gene of *HIF-1 α* by promoter binding (Ebert et al. 1995), in Tibetan goats and Tibetan sheep. Besides, the methylation of these genes were also determined followed the cloning of promoters. These data enhanced the evidence for our understanding about the mechanism underlying hypoxia defense of native plateau animals.

MATERIAL AND METHODS

Animals and sampling

The Animal Care and Use Committee of Southwest University for Nationalities approved all procedures and experiments. Tibetan sheep and Tibetan goats were selected (November 2013) from Zoige County in Sichuan Province at an altitude of 3500 m. Chuanzhong goats were selected from Lezhi County in Sichuan Province at an altitude of 400 m. Small-Tailed Han sheep were selected from Zhumadian City in Henan Province at an altitude of 80 m. Three healthy 3-years-old adult male animals of every breeds were selected (average BW of 56.21 ± 3.55 kg). All the animals were grass grazing and self-feeding. After slaughter, tissue samples (brain, heart, liver, lungs, kidney and muscle) were immediately collected. All samples were rinsed with DEPC (Sigma, USA) sterile water then immediately frozen in liquid nitrogen.

Total RNA extraction and Real-Time quantitative PCR

Total RNA was extracted from tissues (listed above) with Trizol reagent (Invitrogen, Shanghai, China). First-strand cDNA was synthesized from 0.1 to 5 µg of purified total RNA, using the PrimeScript™ RT kit (for perfect Real-time) (Takara, Otsu, Japan) according to the manufacturer's instructions. SYBR® Premix Ex Taq™ II (Takara, Otsu, Japan) was used for measuring the relative expression level of hypoxia genes in various tissues by Real-Time quantitative PCR (RT-qPCR) according to the manufacturer's manual on a CFX96 Real-Time PCR Detection System (BioRad, Hercules, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Zhu et al. 2014) and *β-actin* were used as endogenous references for normalization of targeted mRNA profiles. The primers for RT-qPCR were described as Supplementary Table S1.

Promoters cloning and sequence analyses

DNA was extracted according to previous study (Chen and Leibenguth 1995). The PCR primers and parameters for promoters cloning were described as Supplementary Table S2. Agarose electrophoresis was used for DNA identification (Zhu et al. 2015b). Bisulfite method was used for genome DNA modification. The products of bisulfite modification were then used as PCR template for methylation identification. The primers for core region of promoter cloning were also used for methylation sequencing (Supplementary Table S3).

The promoter sequence was screened by CGD (<http://goat.kiz.ac.cn>) for goat and USUC (<http://genome.ucsc.edu/cgi-bin/hgGateway?>) for sheep. The primers for core region of promoter cloning was designed by Primer Premier 5.0 (Supplementary Table S3). The sequence analyses were performed by BDGP (http://www.fruitfly.org/seq_tools/promoter.html), SoftBerry (<http://linux1.softberry.com/berry.phtml?topic=nsite&group=programs&subgroup=promoter>) and BIMAS (<http://www-bimas.cit.nih.gov/molbio/proscan/>). CpG island and promoter

methylation were predicted using MethPrimer (<http://www.urogene.org/methprimer>).

DNAMAN software was used for vectors and primers removing from the promoter sequences (<http://www.lynnon.com/dnaman.html>). BiQ Analyzer software was used for promoter methylation analysis of the obtained promoter sequences (<http://biq-analyzer.bioinf.mpi-inf.mpg.de/>).

Statistical analyses

SAS9.2 was used for statistical calculations. The RT-qPCR data was analyzed using transcript Ct values normalized with *GAPDH* and *β-actin* as the endogenous control genes (ΔCt). Pfaffl method was used for the difference analyses

$$(\text{Ratio}_{\text{high altitude/low altitude}} = \frac{E_{\text{target gene}}^{\Delta\text{Ct}(\text{high altitude} - \text{low altitude})}}{E_{\text{reference gene}}^{\Delta\text{Ct}(\text{high altitude} - \text{low altitude})}}, E = 10^{-1/\text{slope}}, \text{Real-time PCR}$$

efficiency (E value)) (R.G. Rutledge and C. Coté 2003). Group data for multiple comparisons were analyzed by ANOVA using a general linear model procedure followed by Tukey's test. Significance was established at a $P < 0.05$.

Results

Characterization of hypoxia genes in different tissues of various breeds

For Tibetan sheep, muscle and heart expressed most *FIH-1*, followed by lungs, liver, kidney and brain. Similar expression pattern was observed in the two low-altitude breeds, in which muscle expressed significantly higher *FIH-1* than other tissues, which was followed by kidney, liver, heart, lungs and brain (Figure 1A). Although *FIH-1* was also highly expressed in muscle of Tibetan goats, the lungs expressed most *FIH-1* followed by muscle, kidney, liver, brain and heart.

HIF-1α was highly expressed in liver of all the four breeds, and significantly higher than other tissues in the two sheep breeds. While *HIF-1α* expressed higher mRNA in lungs of Tibetan goats, it gained a relative lower level in lungs of other animals, and lowest in Tibetan

sheep compared with other tissues. The muscle contained lower *HIF-1 α* in all the animals. Besides, *HIF-1 α* was also highly expressed in kidney of all the animals, and significantly higher than other tissues in Chuanzhong goats (Figure 1B).

Heart expressed relative high *HIF-3 α* , actually highest in Tibetan sheep, in different breeds. While kidney expressed lower *HIF-3 α* in high-altitude animals, lowest in Tibetan goats, in low-altitude animals kidney expressed most *HIF-3 α* compared with other tissues. In Tibetan goats, *HIF-3 α* was highly expressed in lungs followed by brain and heart, however in other breeds, it was relative low in lungs. Besides, *HIF-3 α* was relative low in liver and muscle in all the animals (Figure 1C).

EPO expressed similar pattern in all the animals, except for the lowest expression in liver of Tibetan goat despite the altitude discrepancy. Lungs and muscle expressed the lowest *EPO* among different breeds, in which muscle stood the bottom position. Kidney expressed the most *EPO* in high-altitude animals and significantly higher than that in brain. Conversely, the brain tissues of low-altitude animals took the highest *EPO* expression level, thousands fold expression difference was observed in brain tissue comparing with muscle tissue in Chuanzhong goats. A moderate expression level of *EPO* was observed in heart of different breeds (Figure 1D).

In general, for tissue expression, heart contained more mRNA expression of all the four genes in Tibetan sheep. Liver tissue was highly expressed *HIF-1 α* and *EPO* (except in Tibetan goats), and lowly expressed *HIF-3 α* and *FIH-1 α* . Lungs expressed higher hypoxia genes in Tibetan goats and lower in other animals. Kidney contained more *EPO* in all the animals. Muscle tissue was highly expressed *FIH-1 α* , and lowly expressed *HIF-1 α* , *HIF-3 α* and *EPO*. Brain expressed higher *EPO* and lower *FIH-1 α* in all the animals. While both sheep breeds expressed higher *FIH-1 α* , *HIF-1 α* and *HIF-3 α* in muscle, liver and heart respectively, goats got higher mRNA expression of *HIF-3 α* in kidney (Table 1).

Compared with low-altitude animals, *FIH-1 α* expressed highly in lungs and brain, and lowly in liver, kidney and muscle in high-altitude animals. However, different with goat, heart expressed higher *FIH-1 α* in high-altitude sheep (Figure 1E). These discrepancies were observed in the expression of *HIF-1 α* in all the tissues (except in kidney), in which *HIF-1 α* expressed highly in heart, liver, muscle and brain of high-altitude sheep. Especially, the expression of *HIF-1 α* in the heart of low-altitude goats were much lower than that of high-latitude goats (Figure 1F). High-altitude animals expressed higher *HIF-3 α* mRNA expression in lungs and muscle, and lower in kidney and brain. Discrepancies were observed in heart and liver, while the expression of *HIF-3 α* were higher in Tibetan sheep, lower *HIF-3 α* expression was observed in Tibetan goats (Figure 1G). Different with the other three genes, the expression of *EPO* were increased response to high altitude in both goats and sheep, especially in kidney of sheep (667.23 fold) and muscle of goats (1264.19 fold).

Promoters cloning and sequence analyses of hypoxia genes

The promoter sequences of sheep *FIH-1*, *HIF-1 α* , *HIF-3 α* and *EPO* are 1080 bp, 1348 bp, 1608 bp and 2100 bp respectively, with the core regions are 280 bp, 211 bp, 175 bp and 167 bp, including 19, 5, 10 and 5 CpG islands respectively.

The cloned promoter sequences of goat *FIH-1*, *HIF-1 α* , *HIF-3 α* and *EPO* were 1094 bp, 1320 bp, 1890 bp and 1217 bp respectively. Two core regions were observed in the promoter of *FIH-1*, which were 223 bp and 169 bp. This data was followed by 168bp, 267 and 167 bp, contained 24, 5, 12 and 5 CpG islands respectively (Supplementary Table S4).

Methylation analyses of hypoxia genes

For *FIH-1* gene promoter, with the same number of total methylation sites (95 sites), lower methylation ratio was observed in both brain and muscle tissues of low-altitude sheep. While the number of methylated sites were 38 and 33 in brain and muscle in Small-Tailed Han sheep,

65 and 44 sites were observed in Tibetan sheep. Consistent with the situation in sheep, although the total number of methylation was increased significantly up to 120 sites except of Chuanzhong goats (96 sites), higher altitude always indicated more methylated sites. The corresponding number of methylation sites in brain and muscle were 75 and 45 in Chuanzhong goats, and 92 and 82 in Tibetan goats respectively (Table 2).

A total number of 25 methylation sites in *HIF-1 α* promoter core region was shared by goats and sheep. Unlike *FIH-1*, *HIF-1 α* gained more methylated sites in low-altitude animals comparing with plateau animals (Table 3). For details, in liver and muscle tissues, 11 and 15 methylated sites were found in Small-Tailed sheep with the methylation rate were 44% and 60% respectively. These data were replaced correspondingly by 2 and 11 in Tibetan sheep with methylation rate were 8% and 44%. Similar methylation rate was observed in kidney and muscle of Chuanzhong goat than Tibetan goats, containing 13, 16, 4 and 7 methylated sites with the methylation rate were 52%, 64, 16% and 28% respectively.

Consistent with the situation of *HIF-1 α* , *HIF-3 α* promoter gained a total of 50 methylated sites in sheep and 60 methylated sites in goats. For sheep, higher methylation rate in muscle and heart of Small-Tailed Han sheep were observed by 76% and 56%, compared with 64% and 42% in Tibetan sheep respectively. *HIF-3 α* promoter of Chuanzhong goats obtained 71.6% and 55% methylation rate in liver and brain respectively, much higher than 33.3% and 18.3% of Tibetan goats (Table 4).

A total of 25 methylated sites (24 sites in kidney of Chuanzhong goats) were assayed in the promoter of *EPO* gene in both sheep and goats. Much more methylated sites were observed in kidney and muscle of Small-Tailed Han sheep, recorded as 14 and 20 respectively, compared with 3 and 13 methylated sites in Tibetan sheep, with the calculated methylation rate was 56%, 80% and 3% and 13% respectively. Although no significant methylated rate change was observed in the muscle of Chuanzhong goat and Tibetan goat, despite a slightly

higher data in Chuanzhong goats, the promoter of *EPO* showed much higher methylation rates (54.2%) than Tibetan goats (4%) (Table 5).

DISCUSSION

Qinghai-Tibetan plateau is the highest plateau raises some special animal species tolerant to the extreme hypoxia environment, including Yak, Tibetan goat, Tibetan sheep, Tibetan chicken and Tibetan pig and so on. These plateau animals have become an essential part for the life of Tibetan people. Undoubtedly the role of *FIH-1* (Kiriakidis et al. 2015), *HIF-1 α* (Kitajima and Miyazaki 2013), *HIF-3 α* (Augstein et al. 2011; Zhang et al. 2012) and *EPO* (Ozawa et al. 2010) have been well-established in human, mice or fish. However, these studies could not shed light on their physiological roles in hypoxia resistance in high-altitude plateau. The present study is novel in that we firstly investigated the tissue expression discrepancy of hypoxia genes between high-altitude and low-altitude animals, especially for higher expression of *EPO* in higher-altitude animals, and observed higher promoter methylation rates of *HIF-1 α* , *HIF-3 α* and *EPO*, lower rate of *FIH-1* in high-altitude species, indicated that the expression of hypoxia genes and the promoter methylation may partly illuminate the resistance of plateau species.

HIF-1 is a heterodimer consisting of *HIF-1 α* and *HIF-1 β* subunits (Wang et al. 1995; Wang and Semenza 1995). It plays an essential role in cellular and systemic oxygen homeostasis (Iyer et al. 1998). *HIF-1* activates transcription of genes whose protein products either function to increase O₂ availability by promoting erythropoiesis (e.g., erythropoietin) and angiogenesis

or mediate adaptive intracellular responses to O₂ deprivation such as increased glycolytic metabolism (e.g., glucose transporters and glycolytic enzymes) (Semenza 1999). Whereas *HIF-1 α* is constitutively expressed, *HIF-1 β* mRNA expression, protein half-life, and transactivation domain function are all regulated by the cellular O₂ concentration in vivo

(Semenza 1999). Previous study showed that *FIH-1* interacted with *HIF-1 α* and von Hippel-Lindau (*VHL*) to mediate repression of *HIF-1* transcriptional activity because of the asparaginyl hydroxylase enzyme activity (Mahon *et al.*, 2001; Lando *et al.*, 2002), and had been well recognized as the main regulator of *HIF-1*. Based on the literatures above, comparing the mRNA expression of *FIH-1* and *HIF-1 α* between high-altitude and low-altitude species was hypothesized to be an available method for probing their role in hypoxia resistance. In the present study, the similar higher *FIH-1* expression of Tibetan sheep and Tibetan goats in lungs may explain their adaptation to low-oxygen concentration, this was consistent with previous results that *FIH-1* was associated with the induction of vascular endothelial growth factor (*VEGF*) mRNA expression in response to hypoxia via the control of *HIF-1 α* (Carmeliet *et al.* 1996; Iyer *et al.* 1998; Maltepe *et al.* 1997; Ryan *et al.* 1998). Besides, the higher expression of *HIF-1 α* in lungs also enhanced the effect of *FIH-1* in Tibetan goat. Converse expression pattern of *FIH-1* was determined in heart of sheep and goats. As a result, much lower expression of *HIF-1 α* was corresponded. Although the divergence between different species was supposed to be a reasonable factor, a future study was still necessary for an exact explanation between sheep and goats.

The human *HIF-3 α* locus is subject to extensive alternative splicing, leading to at least seven variants. All these variants were found to interact with *HIF- β* , *HIF-1 α* and *HIF-2 α* . Interaction of the *HIF-3 α* variants with *HIF-1 α* and *HIF-2 α* inhibited the nuclear translocation of both, indicated the negative function for hypoxia response (Pasanen *et al.* 2010). Unexpectedly, siRNA knock-down of the endogenous *HIF-3 α* variants led to downregulation of certain HIF target genes, overexpression of individual long *HIF-3 α* variants upregulated certain HIF target genes in a variant and target gene-specific manner in which *HIF- β* was not a limiting factor (Heikkilä *et al.* 2011). Corresponding to the versatile function, in the present study, *HIF-3 α* expressed similar pattern in lungs, kidney, muscle and brain between goats and

sheep responded to hypoxia environment, however in heart and liver goats and sheep shown different *HIF-3 α* expression alteration in high-altitude area. In rats, under acute hypoxia (12% O₂, two hours) *HIF-3 α* mRNA expression was strongly elevated in heart, lung, kidney and muscle tissues (Drevytska et al. 2012). Marc *et al.* also showed that rats *HIF-3 α* mRNA levels increased in brain, lung, heart, kidney, and liver after two hours of hypoxia (Maynard et al. 2007). These data were consistent with the higher expression of *HIF-3 α* in heart, liver, lungs and muscle of Tibetan sheep in the present study. However, different with previous study, although lungs and muscle expressed higher expression of *HIF-3 α* , other tissues obtained lower expression in Tibetan goats. More controlled functional experiments using RNA interference and overexpression in different tissues of Tibetan goats and Tibetan sheep may be beneficial for our understanding about the role of *HIF-3 α* in response to hypoxia in the future.

It is well known that *EPO* plays an essential role in the delivery of oxygen from the lung to every organ, and increased response to hypoxia (Suzuki 2015). Besides, independent of hematopoietic activity, administration of *EPO* improves cardiac contractility in post-hypoxic mice (Sterin-Borda et al. 2003), represses cell death of the myocardium in a rabbit AMI model (Parsa et al. 2003), and is a promising treatment for acute myocardial infarction (Ozawa et al. 2010). Enhanced expression of *EPO* gene promoted the differentiation (Shoemaker et al. 1986), proliferation, migration and invasion via the p38MAPK/AP-1/MMP-9 pathway in vascular smooth muscle cells (Park et al. 2015). Although no correlation was observed between *EPO* expression and intratumoral hypoxia in cervical cancers (Leo et al. 2006), in the present study *EPO* gained much higher expression in all the tissues detected of Tibetan goats and Tibetan sheep consistent with the previous data in brain (Marti 2004). We also knew that *HIF-1* and *HIF-2* regulated hypoxia resistance partly via the control of *EPO* (Marti 2004). Thus, the high expression of *EPO* may give the final effect induced by the alteration of hypoxia genes in a low-oxygen press environment.

It is well believed that three inner reference genes were the optimum choice for normalizing the results of RT-qPCR in bovine (Kadegowda et al. 2009). In cow and goat Eukaryotic translation initiation factor 3K (*EIF3K*), Topoisomerase II-beta (*TOP2B*) and Ubiquitously expressed transcript (*UXT*) were proposed as the most stable reference genes (Bonnet et al. 2013). Our previous study showed that *GAPDH* (Lin et al. 2013; Zhu et al. 2015a; Zhu et al. 2014) and *β-actin* (Zhang et al. 2013) were also suitable as reference genes in goat (Zhang et al. 2013). Obviously more reference genes are also needed for identifying the hypoxia genes expression in the future. In the present study, we investigated the expression profiles of hypoxia genes in various tissues of four breeds respectively using a relative quantification method ($2^{-\Delta\Delta C_t}$). However, due to the expression differences of reference genes between different breeds, and also the differences of reference tissues, the relative quantification method was not suitable for comparing the expression of hypoxia genes among different breeds. To solve the problem, an absolute quantification method is necessary for comparing the expression discrepancies of hypoxia genes among different breeds in the future.

We also recognized that the number of animals, three for each species, in the present study is small. However, considering that two species, including six animals (three Tibetan sheep and three Tibetan goats), were selected from high and low altitude respectively, our data may partly explain the mRNA expression alteration of hypoxia genes response to extremely low oxygen environment. Actually, both of goats and sheep results showed that *EPO* may sensitive to hypoxic environment. In addition, six goats (three Tibetan goats and three Chuanzhong goats) and six sheep (three Tibetan sheep and three Small-Tail Han sheep) were also suitable for analyzing the discrepancy between different species. However, five or even more animals for each breeds were encouraged to confirm the results in the future.

Although higher methylation rate in the promoter was believed to suppress the mRNA

expression, here there was no significant association between gene expression and methylation. An exact mechanism remains unclear, more research about how the methylation of gene promoter affects mRNA expression will be beneficial for understanding the role of methylation in hypoxia resistance. We also recognized the limitation of the methylation analyses in only two tissues in each breeds. The results from the same tissues between Small-Tail Han sheep and Tibetan sheep, Chuanzhong goat and Tibetan goat in the four genes also told us that plateau animal contained more methylation rate compared with low-altitude species except for *FIH-1*. Undoubtedly, a methylation analyses of the full-length genes are necessary for better understanding the transcription regulation response to hypoxia in the future.

CONCLUSION

We firstly identified the mRNA expression of *FIH-1*, *HIF-1 α* , *HIF-3 α* and *EPO* in heart, liver, lungs, kidney, muscle and brain of four different species, including Tibetan sheep, Tibetan goat, Small-Tail Han sheep and Chuanzhong goat. *EPO* was highly expressed in all the tissues detected in Tibetan goat and sheep, and was predicted to play a crucial role in responding to hypoxia. The promoter length of *FIH-1*, *HIF-1 α* , *HIF-3 α* and *EPO* are cloned, and also identified the core region and CpG islands in goats and sheep. Higher methylation rate was observed in *HIF-1 α* , *HIF-3 α* and *EPO*, while lower methylation rate hit on *FIH-1* in plateau species. These results provide a basal information for the functional research of hypoxia genes, and will certainly facilitate our understanding about the response mechanism to hypoxia of plateau animals.

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Table 1 Tissues expression arrange of hypoxia genes in different species

	FHI-1	HIF-1 α	HIF-3 α	EPO
Tibetan sheep	Muscle>Heart>Lungs	Liver>Brain>Heart	Heart>Liver>Kidney	Kidney>Brain>Liver
	>Liver>Kidney>Brain	>Muscle>Kidney>Lungs	>Lungs>Muscle>Brain	>Heart>Lungs>Muscle
Tibetan goat	Lungs>Muscle>Kidney	Lungs>Kidney>Liver	Lungs>Brain>Heart	Kidney>Heart>Lungs
	>Liver>Brain>Heart	>Brain>Heart>Muscle	>Muscle>Liver>Kidney	>Brain>Muscle>Liver
Small-Tailed Han sheep	Muscle>Kidney>Liver	Liver>Kidney>Lungs	Kidney>Heart>Brain	Brain>Liver>Kidney
	>Heart>Brain>Lungs	>Brain>Heart>Muscle	>Lungs>Muscle>Liver	>Heart>Lungs>Muscle
Chuanzhong goat	Muscle> Kidney>Liver	Kidney>Liver>Heart	Kidney>Heart>Brain	Brain>Kidney>Liver
	>Heart>Lungs>Brain	>Lungs>Brain>Muscle	>Lungs>Liver>Muscle	>Heart>Lungs>Muscle

Table 2 Methylation analysis of core region of *FIH-1* promoter

Tissues	Small-Tailed Han sheep		Tibetan sheep		Chuanzhong goat		Tibetan goat	
	Brain	Muscle	Brain	Muscle	Brain	Muscle	Brain	Muscle
Number of methylated sites	38	33	65	44	75	45	92	82
Number of unmethylated sites	57	62	30	51	45	51	28	38
Total number	95	95	95	95	120	96	120	120
Methylation rate %	40	34.7	68.4	46.3	62.57	46.9	76.6	68.3

Table 3 Methylation analysis of core region of *HIF-1 α* promoter

Tissues	Small-Tailed Han sheep		Tibetan sheep		Chuanzhong goat		Tibetan goat	
	Liver	Muscle	Liver	Muscle	Kidney	Muscle	Kidney	Muscle
Number of methylated sites	11	15	2	11	13	16	4	7
Number of unmethylated sites	14	10	23	14	12	9	21	18
Total number	25	25	25	25	25	25	25	25
Methylation rate %	44	60	8	44	52	64	16	28

Table 4 Methylation analysis of core region of *HIF-3 α* promoter

Tissues	Small-Tailed Han sheep		Tibetan sheep		Chuanzhong goat		Tibetan goat	
	Muscle	Heart	Muscle	Heart	Liver	Brain	Liver	Brain
Number of methylated sites	38	28	32	21	43	33	20	11
Number of unmethylated sites	12	22	18	29	17	27	40	49
Total number	50	50	50	50	60	60	60	60
Methylation rate %	76	56	64	42	71.6	55	33.3	18.3

Table 5 Methylation analysis of core region of *EPO* promoter

Tissues	Small-Tailed Han sheep		Tibetan sheep		Chuanzhong goat		Tibetan goat	
	Kidney	Muscle	Kidney	Muscle	Kidney	Muscle	Kidney	Muscle
Number of methylated sites	14	20	3	13	13	18	1	17
Number of unmethylated sites	11	5	22	12	11	7	24	8
Total number	25	25	25	25	24	25	25	25
Methylation rate %	56	80	12	52	54.2	72	4	68

LEGENDS

Fig. 1 Tissue expression of hypoxia genes in different species. The mRNA expression of *FIH-1* (A), *HIF-1 α* (B), *HIF-3 α* (C) and *EPO* (D) in heart, liver, lungs, kidney, muscle and brain among Tibetan sheep, Tibetan goat, Small-Tail Han sheep and Chuanzhong goat. mRNA expression of *FIH-1* (E), *HIF-1 α* (F), *HIF-3 α* (G) and *EPO* (H) of Tibetan sheep and Tibetan goat relative to Small-Tailed Han sheep and Chuanzhong goat respectively. *GAPDH* and *β -actin* as endogenous control for RT-qPCR analyses. The data of each experimental group represents the mean $\pm$ SD of three repeats where RT-qPCR samples were measured in triplicates. The significance was performed at $P < 0.05$.

196x222mm (300 x 300 DPI)