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The role of sex-determining region Y-box 6 in melanogenesis in alpaca melanocytes

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ABSTRACT

Background: Sex-determining region Y-box (SOX) proteins function as transcriptional regulators. The derivation of melanocytes from nerve crest cells has been reported to depend on SOX proteins, including SOX10 and SOX5. Whether SOX6 is expressed and has a functional role in melanocytes is unknown.
Objective: We aimed to study the effect of transcription factor SOX6 on melanogenesis in alpaca melanocytes.
Methods: We verified the role of SOX6 in melanogenesis by overexpressing and inhibiting SOX6 in melanocytes. Co-immunoprecipitation (co-IP) experiments were performed to further explore the function of SOX6 in melanogenesis and its mechanism of melanin production. We found that SOX6 interacted with cyclin-dependent kinase CDK5, β-catenin, and Cyclin D1.
Results: Bioinformatics analysis suggested that SOX6 has a phosphorylation site for CDK5, which regulates melanogenesis, suggesting that SOX6 might play a role in melanogenesis. Co-IP experiments indicated that SOX6 interacted with CDK5, β-catenin, and Cyclin D1. Quantitative real-time polymerase chain reaction and western blot analyses of SOX6-overexpressing melanocytes revealed increased mRNA and protein expression of Cyclin D1, CDK5, microphthalmia transcription factor (MITF), tyrosinase (TYR), tyrosine related protein-1 (TYRP1), and dopachrome-tautomerase (DCT), whereas β-catenin levels decreased in SOX6-overexpressing melanocytes. The opposite results were observed upon SOX6 knockdown. The melanin content was significantly increased or decreased, respectively, by SOX6 overexpression or knockdown.
Conclusion: Our results suggest that SOX6 might enhance melanogenesis by binding with β-catenin to increase Cyclin D1 and MITF expression.

1. Introduction

The sex-determining region Y-box (*SOX*) family consists of 20 genes [1]. These genes encode transcriptional factors with a high-mobility group (HMG) box DNA-binding domain, which is very similar to that of the sex-determining region (Sry) protein [2]. Based on the sequence identity inside and outside of this domain, the SOX genes are classified into 8 groups, named as SOXA through SOXH. *SOX6* belongs to the SOXD group in most vertebrates, together with *SOX5* and *SOX13* [2].

The expression of SOX proteins, which function as transcriptional regulators [3], is tightly regulated by many signaling pathways [4]. Their transcriptional activities are subject to post-translational modification and sequestration mechanisms [5]. In brain tissue, the nuclear transcription factor SOX6 is a direct nuclear target for phosphorylation by CDK5 [6]. In chondrocytes, SOX5 and SOX6 expression is induced by SOX9 [7]. Once induced, SOX5 and SOX6 cooperate with SOX9 to activate common target genes such as *COL2A1* [8], and hence, SOX5 and SOX6 increase the transcriptional activity of their own inducer, SOX9.

Melanogenesis depends on SOX proteins [9]. SOX10 is responsible for activating expression of the melanocyte-specific isoform of *MITF-M*gene. MITF is the key regulator of the transcription of melanogenic enzymes during melanogenesis in melanocytes [10], includingTYR, TYRP1, and DCT [11]. It has been reported that SOX5 binds to SOX10 response elements [12] to participate in *MITF* regulation in human melanoma cells [13]. In addition, competition for binding sites may be one of the ways in which SOX5 interferes with the function of SOX10 [12]. *SOX6* is expressed in B16 melanoma cells, and SOX6 knockdown does not

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affect MITF levels in B16 cells [12]. SOX6 represses Cyclin D1 gene expression in pancreatic β cells and ε-globin expression during definitive erythropoiesis [14]. Moreover, SOX6 can physically interact with β-catenin in pancreatic β cells [14]. *SOX5* and *SOX6* show high sequence similarity in their respective HMG boxes, suggesting that they might perform similar function in some cells.

In this study, the function of SOX6 was investigated by gain and loss of function experiments in alpaca melanocytes. We also uncovered the pathway whereby SOX6 regulates melanogenesis. In addition, we found that SOX6 regulates melanogenesis by binding with β-catenin to regulate Cyclin D1 in alpaca melanocytes. We believe that our findings will be particularly valuable to researchers with an interest in hair color formation in fiber-producing animals and melanoma biology.

2. Materials and methods

2.1. Cell culture of alpaca melanocytes

The alpaca melanocytes used in this study were obtained from an established stock, maintained as previously described [15]. For gene cloning and immunocytochemistry of SOX6, melanocytes from passage 5 were recovered and cultured in melanocyte medium until the cells were >80% confluent. Some of the cells were flash-frozen in liquid nitrogen and stored at −80 °C for subsequent RNA extraction, while the remaining cells were trypsinized, plated on cover slips, and cultured for 3 days before detecting SOX6 expression by immunocytochemistry.

2.2. Gene cloning and construction of a SOX6-expression vector

The complete coding sequences (CDSs) of wild-type alpaca *SOX6* and *SOX6* mutation at the phosphorylation site T119 (T119 as predicted using Scansite software:http://scansite.mit.edu/motifs-can_seq.phtml) were amplified by reverse transcription polymerase chain reaction (PCR) using the kit (Bimake, Shanghai, China) and gene-specific primers (Table 1) and subcloned into a pMD-19T vector to yield the plasmids pMD-19T-SOX6 and pMD-19T-SOX6-mut. The *SOX6* inserts were liberated from the pMD-19T-SOX6 and pMD-19T-SOX6-mut plasmids using endonucleases *Kpn*I and *Xba*I and ligated into a pcDNA3.1 expression vector to construct pcDNA3.1-SOX6 and pcDNA3.1-SOX6-mut. The sequence of each construction was verified by restriction digestion and DNA sequencing. Based on the alpaca *SOX6* CDS sequence, 3 *SOX6* small interfering RNAs (siRNAs) designed to target alpaca *SOX6* mRNA, and a negative control (NC) siRNA (scrambled) were obtained from Sangon Biotech (Shanghai, China).

2.3. Overexpression of SOX6 in alpaca melanocytes

Alpaca melanocytes were cultured in complete melanocyte culture medium (ScienCell Research Laboratories, Inc., San Diego, CA) in a 6-well plate (1×10^5 cells/well) for 24 h to approximately 70% confluence. On day 2, 1.5 μg of the pcDNA3.1-SOX6 or pcDNA3.1-SOX6-mut plasmid in serum-free medium was mixed with 5 μL of DNA Fectin transfection reagent (Tiangen Biotech, Beijing, Co. Ltd. China) and incubated at room temperature for 20 min to allow DNA–liposome complexes to form. The growth medium of the cultured melanocytes was removed and replaced with 800 μL serum-free medium and either the pcDNA3.1-SOX6 or pcDNA3.1-SOX6-mut DNA–liposome complex. Control cells were transfected as described above using the NC (NC is a sequence that disrupts the base sequence and must not be homologous to other genes.). After incubation at 37 °C for 20 h, the medium containing each transfection solution was removed, and 2 mL complete growth medium (with serum) was added to each well. The melanocytes were incubated at 37 °C for 3 days. Next, the cells were trypsinized, and cell pellets were frozen at −80 °C for subsequent analysis or used immediately to prepare slide smears for immunocytochemical experiments.

Table 1
Sequences for the qRT-PCR primers and siRNAs used in this study.

Primer name	Primer sequence 5′–3′	Annealing temperature
SOX6-F	AAGATGCTGACTGGGACA	54 °C
SOX6-R	GGTGAGGTAGAGGTATTTCG	
β-catenin-F	GACCCTGCCATCTGTGC	58 °C
β-catenin-R	CGGGTGGAGGAGTTTCA	
CDK5-F	GTGGCTCTGAAACGGGTGA	58 °C
CDK5-R	GCCTGACGATGTTCTTGTG	
Cyclin D1-F	TGCGAGCCATGCTCAAGACGG	52 °C
Cyclin D1-R	TCTTAGAGGCCACGAACATGCAGGT	
MITF-F	TCCCAAGTCAAATGATCCAG	56 °C
MITF-R	GAGCCTGCATTTCAAGTTCC	
TYR-F	GCTTTAGCAACTTCATGGGA	58 °C
TYR-R	CTTGTTCTTCTCTGGGACAC	
TYRP1-F	GCTCAGTGCTTGGAAGTTGGT	60 °C
TYRP1-R	AGTTTGTCCTCCAGTTCCGTTTAG	
DCT-F	TGCTTTGCCCTACTGGAAC	58 °C
DCT-R	ATCAGAGTCGATCGTCTG	
18S-F	GAAGGGCACCACCAGGAGT	50–60 °C
18S-R	CAGACAAATCACTCCACCAA	
SOX6-siRNA-1-F	GCUACCAACACUUGUCAAUTT	54 °C
SOX6-siRNA-1-R	AUUGACAAGUGUUGGUAGCTT	
SOX6-siRNA-2-F	GCCAACAGCAAGAACAGAUTT	54 °C
SOX6-siRNA-2-R	AUCUGUUCUUGCUGUUGGCTT	
SOX6-siRNA-3-F	GGAGCAAAGAUGCCAUCAATT	54 °C
SOX6-siRNA-3-R	UUGAUGGCAUCUUUGCUCCTT	
siRNA −NC-F	UUCUCCGAACGUGUCACGUTT	54 °C
siRNA −NC-R	ACGUGACACGUUCGGAGAATT	
SOX6-mut-F	ACTTTTGGAGCCCCAGAACGCCGCA	60 °C
SOX6-mut-R	TGCGGCGTTCGGGGCTCCAAAAGT	

SOX6 = sex determining region Y-box 6; F = forward;R = reverse; CDK5 = cyclin dependent kinase 5; MITF = microphthalmia transcription facto; TYR = tyrosinase; DCT = dopachrome tautomerase.

2.4. Silencing SOX6 expression in alpaca melanocytes

Six microliters of siRNA was added to 100 μL siRNA transfection medium (Santa Cruz Biotechnology), followed by 6 μL siRNA transfection reagent (Santa Cruz Biotechnology). Melanocytes were plated and cultured as described above until they were 70% confluent. Cells were then transfected with SOX6 siRNA or the NC siRNA. The cells were incubated for 5–7 h at 37 °C with 5% CO_2. Then, 1 mL normal melanocyte growth medium containing serum and antibiotics was added, and the cells were incubated for an additional 18–24 h.

2.5. Total RNA isolation and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was isolated from cultured alpaca melanocytes using the TRIzol reagent (Invitrogen). Approximately 1 μg total RNA per sample was converted to cDNA. qRT-PCR analysis of the mRNA expression levels of the *SOX6*, *CDK5*, Cyclin D1, β-catenin, *TYR*, *TYRP1*, and *DCT* genes in cultured melanocytes was conducted using a SYBR Green-based detection system (TaKaRa, Dalian, China) in association with the comparative-threshold cycle method, with normalization relative to the abundance of 18S rRNA. Each 25-μL PCR was performed according to the manufacturer's instructions (Invitrogen) with the following the cycling conditions: 95 °C for 2 min, 45 cycles of 57 °C for 25 s and 95 °C for 15 s. All reactions were performed in 4 repetition in a Stratagene Mx3005P real-time PCR instrument.

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