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Stable transfection and identification of a hair follicle-specific expression vector of IGFBP-5 in goat fetal fibroblasts

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ABSTRACT. The insulin-like growth factor-binding protein-5 (IGFBP-5) is one of the 6 members of the IGFBP family and is involved in the regulation of cell growth, apoptosis, and other IGF-stimulated signaling pathways. To determine the significance of IGFBP-5 in the Inner Mongolia Cashmere goat (*Capra hircus*), a hair follicle-specific expression vector of IGFBP-5, pCDsRed2-K-IGFBP5 (6.7 kb), was constructed by cloning *IGFBP-5* downstream of the keratin-association protein (KAP)6-1 promoter and inserting this fragment into pCDsRed2, which contains a red fluorescent protein (DsRed) expression unit. Inner Mongolia Cashmere goat fetal fibroblast (GFb) cells were transfected with the expression vector by using Lipofectamine™ 2000. Cell clones that stably expressed red fluorescence were obtained after selection with Geneticin (G418). The transgene in the cell clones was examined by polymerase chain reaction to verify that exogenous DNA (pKAP6-1 and IGFBP-5) had integrated stably into GFb cells. These data suggest

that this method can be used for the construction of a hair follicle-specific expression vector for functional genetic analyses and for obtaining stable transfection donor cells for nuclear transfer.

Key words: Inner Mongolia Cashmere goat; IGFBP-5; Hair follicle-specific expression vector; Stable transfection

INTRODUCTION

Insulin-like growth factor-binding proteins (IGFBPs) constitute a family of secreted proteins that bind insulin-like growth factors I and II (IGF-I and -II). IGFBPs modulate the effects of IGFs on target cells (James et al., 1993). IGFBP-5 is a critical, and the most evolutionarily conserved, member of the IGFBP family (Allander et al., 1994; Beattie et al., 2006), and is involved in many processes, including bone, ovary, mammary gland, and kidney physiology (Kelley et al., 1996). IGFBP-5 primarily mediates cell growth, suppressing or inducing proliferation (Andress and Birnbaum, 1992; Ewton et al., 1998; Meadows et al., 2000; Schneider et al., 2001).

IGFBP-5 is also associated with the development and differentiation of hair; however, the underlying mechanisms have not yet been examined. Hair features are affected by many factors, the two most significant of which being the follicle's capacity and the hair growth cycle. IGF-I signaling is an important mitogenic and morphogenetic regulator in hair follicle biology (Weger and Schlake, 2005). IGFBP-5 regulates the hair shaft structure and follicle identity (Schlake, 2005b), and was identified as the first molecular marker in zigzag hair follicles (Schlake, 2005b). In 2006, Schlake reported Krox-20 as a molecular marker, whose expression in the proximal follicle was restricted to zigzag hair follicles, implicating it as a nodal point of the fibroblast growth factor and IGF signaling pathways, which control IGFBP-5 expression and, therefore, possibly affect the periodicity of the zigzag hair phenotype.

IGFBP-5 has been cloned in humans, mice, and rats, but little is known about its function in small ruminants. Specifically, the function of *IGFBP-5* in goat cells is currently unknown. In this study, we constructed a hair follicle-specific expression vector of *IGFBP-5* and stably transfected it into goat fetal fibroblast (GFb) cells. Our data provide a method of constructing a hair follicle-specific expression vector for functional genes and for obtaining stable transfection donor cells for nuclear transfer. Furthermore, results of this study will facilitate further research of the function of IGFBP-5 in hair follicle development and hair features.

MATERIAL AND METHODS

Cell culture conditions

Inner Mongolia Cashmere GFb cells were maintained as monolayer cultures in Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12; Gibco, Paisley, UK), supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT, USA), 100 U/mL penicillin G, and 100 mg/mL streptomycin (Sigma-Aldrich, Inc., St. Louis, MO, USA). Cell cultures were maintained and incubated at 37°C in humidified air with 5% CO_2.

Construction of pCDsRed2-IGFBP5

The pDsRed2-1 vector, containing a red fluorescent protein (DsRed), was used to generate pCDsRed2, in which the cytomegalovirus (CMV) promoter was placed upstream of the DsRed2 gene (Guo et al., 2009). pMD19T-IGFBP5 was digested with S*al*I and K*pn*I, and an IGFBP-5 fragment was cloned into pCDsRed2 to generate pCDsRed2-IGFBP5. Recombinant plasmids were verified by restriction analysis on a UV transilluminator (UVItec, London, UK) after electrophoresis.

Construction of the skin-specific expression vector, pCDsRed2-K-IGFBP5

pMD19TK was digested with *Eco*RI and S*al*I (TaKaRa, Dalian, China) to yield the keratin-associated protein (KAP)6-1 fragment, which was cloned into pCDsRed2-IGFBP5 to generate pCDsRed2-K-IGFBP5. The recombinant plasmid was verified by restriction analysis on a UV transilluminator (UVItec) after electrophoresis. The map of pCDsRed-K-IGFBP5 is shown in Figure 1.

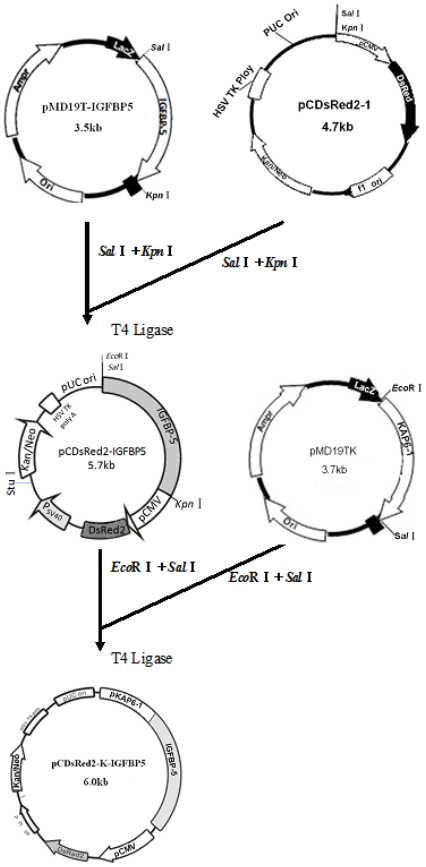

Figure 1. Diagram of hair follicle-specific expression vector pCDsRed-K-IGFBP5.

Transfection *in vitro*

Four micrograms pCDsRed-K-IGFBP5 was added to 8 µL Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) and mixed with 5 mL serum-free media. GFb cells were grown from 60 to 70% confluence on 6-well plates, and then incubated with this media for 6 h at 37°C and 5% CO_2; empty vector and untransfected cells were used as controls. The medium containing Lipofectamine™ 2000 was removed, and the cells were incubated in DMEM/F12 medium with 10% FBS. The cells were selected with 800 µg/mL Geneticin (G418; Hyclone Laboratories) 48 h after transfection, and the medium was replaced on alternate days until cells fluoresced red and green.

Verification of the stably transfected vector in GFbs

Genomic DNA from stably expressing GFb cells, empty vector-transfected cells, and untransfected cells were examined by polymerase chain reaction (PCR). Inner Mongolia Cashmere goat genomic DNA fragments were amplified using a pair of specific primers based on goat pCDsRed2-K-IGFBP5-forward primer: 5'-CACACCCACACTGAGAGC-3'; reverse primer: 5'-CGATCTCGAACTCGTGGC-3'. The start and end positions are shown in Figure 2.

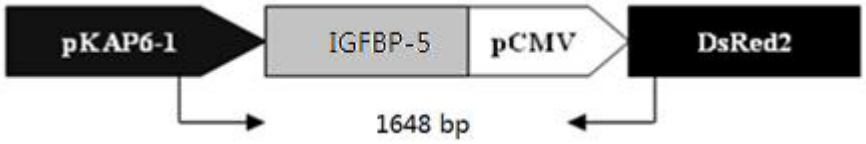

Figure 2. Schematic of PCR.

The PCR was run at 94°C for 5 min; followed by 35 cycles of 94°C for 1 min, 50°C for 2.5 min, and 72°C for 2.5 min; and 72°C for 10 min. PCR mixtures (25 µL) contained 0.25 µL TaKaRa LA Taq (TaKaRa), 2.5 µL 10X LA buffer (TaKaRa), 4 µL 2.5 mM of each dNTP, 0.5 µL template DNA, 1 µL 10 µM of each primer mixture, and 16.75 µL d_3H_2O. PCR products were electrophoresed, and photographs were taken on a UV transilluminator (UVItec). The predicted product length was 1648 bp.

RESULTS

Construction and identification of expression vectors

To express IGFBP-5, the recombinant plasmid pCDsRed-K-IGFBP5 was constructed, based on the pCDsRed2 backbone. The IGFBP-5 fragment was released from S*al*I- and K*pn*I-digested pMD19T-IGFBP5. Then, the IGFBP-5 expression fragment was cloned into pCDsRed2-1 to generate pCDsRed2-IGFBP5. pMD19TK and pCDsRed2-IGFBP5 were digested with *Eco*RI and S*al*I, and the KAP6-1 fragment was cloned into pCDsRed2-IGFBP5. The recombinant plasmids were transformed into *Escherichia coli*. pCDsRed-K-IGFBP5 was identified by restriction analysis (Figure 3). The restriction analysis of pCDsRed2-IGFBP5 is shown in Figure 3A; the IGFBP5 fragment was 816 bp. The restriction analysis of pCDsRed-K-IGFBP5 is shown in Figure 3B; the KAP6-1 fragment was 1052 bp long. Finally, the plasmids were sequenced, and the correct clones were selected.

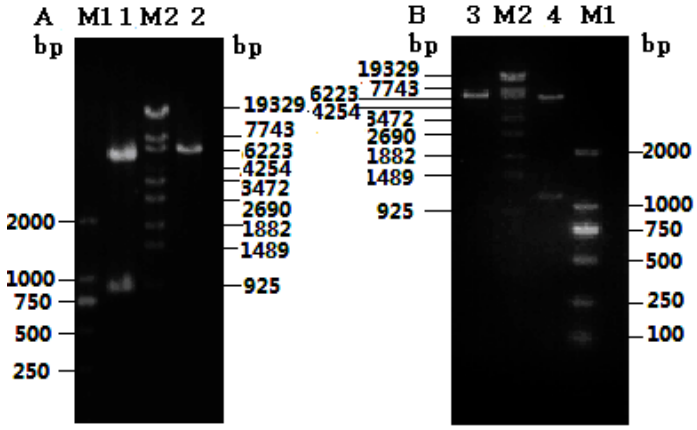

Figure 3. Restriction analysis of pCDsRed2-IGFBP5 (**A**) and pCDsRed2-K-IGFBP5 (**B**). A: *lane M1* = DL2000 marker; *lane 1* = pCDsRed2-IGFBP5 digested by *Sal*I + *Kpn*I; *lane M2* = λ-EcoT14 I-digested DNA marker; *lane 2* = pCDsRed2-IGFBP5 digested by *Sal*I; B: *lane 3* = pCDsRed2-K-IGFBP5 digested by *Sal*I; *lane M2* = λ-EcoT14 I digest DNA marker; *lane 4* = pCDsRed2-K-IGFBP5 digested by *Eco*RI+ *Sal*I; *lane M1* = DL2000 marker.

Evaluation of stability for GFbs

Lipofectamine™ 2000 was used to transfect pCDsRed-K-IGFBP5. An empty vector and untransfected cells were used as controls. The expression of DsRed was visualized under a fluorescence microscope (Olympus IX71, Japan), and photographs were taken using a digital camera. Red fluorescence of the transfected cells was observed 48 h after transfection. The transfection rate was 9% (Figure 4A and B). There were dense areas of fluorescence after selection with G418 for 2 weeks (Figure 4C and D).

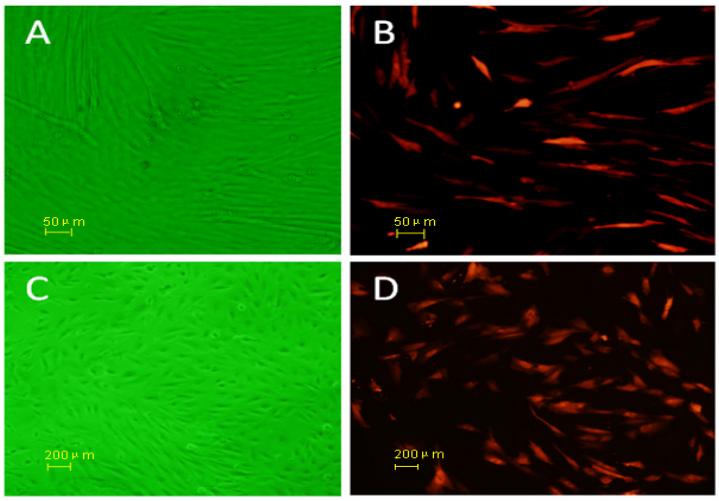

Figure 4. Cells 48 h and 15 days after transfection. **A.** Bright-field images of cells (after 48 h). **B.** Red fluorescence of transfected cells (after 48 h). **C.** Bright-field images of cells (after 15 days). **D.** Red fluorescence of transfected cells (after 15 days).

PCR identification of the stably transfected vector in GFbs

Using primers that spanned KAP6-1 to DsRed2 and genomic DNA from GFb cells as a template, a 1648-bp fragment was amplified; no such fragment was obtained in the control groups (Figure 5). PCR of the target segment in GFb cells demonstrated that goat *IGFBP-5* had been transfected into GFb cells and that the transfectants could be used as nuclear donors of transgenic somatic cells.

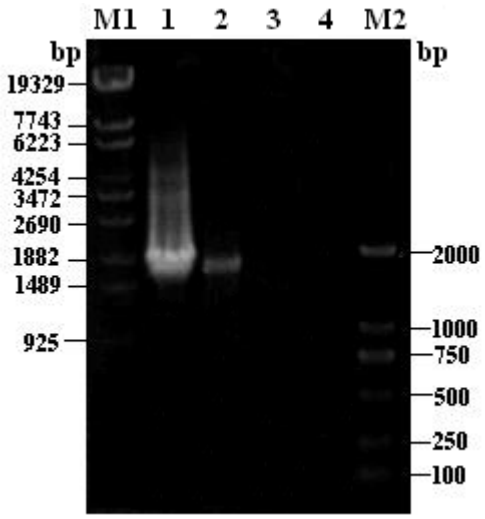

Figure 5. Identification of transgenic cell clones by PCR. *Lane M1* = λ-Ecot14 I-digested DNA marker; *lane 1* = pCDsRed2-K-IGFBP5 plasmid control; *lane 2* = pCDsRed2-K-IGFBP5-transfected cells; *lane 3* = pCDsRed2-transfected cells; *lane 4* = untransfected cell control; *lane M2* = DL2000 marker.

DISCUSSION

IGFBP-5 is 17 kb in length and is located on chromosome 1 in mice (Kou et al., 1994). In humans, it is 33 kb in length, and lies on chromosome 2 (Allander et al., 1994). IGFBP-5 has a cysteine-rich *N*-terminal domain (12 cysteines) and a cysteine-rich *C*-terminal domain (6 cysteines) (Duan, 2002). The effect of IGFBP-5 on cell survival appears to be based on interactions with specific soluble or cell-associated ligands (Schneider et al., 2002; Beattie et al., 2006; Hung et al., 2008). The *N*- and *C*-terminal domains are highly conserved among members of the IGFBP family across species (Duan, 2002). The *N*-terminal domain of IGFBP-5 contains the IGF-1-binding region and caveolin-binding sites (Ravid et al., 2008). The *C*-terminal domain cannot bind IGF-1 but can affect the affinity to IGF-1 (Shand et al., 2003). A highly basic domain in the *C*-terminal end of IGFBP-5 (AA220-237) contains a functional nuclear localization sequence (Schedlich et al., 1998).

The hair follicle is primarily an ectodermal derivative with a complex epithelial structure, and it is generated by common progenitor cells. The dermal papilla, which is of mesenchymal origin, can control morphogenesis, hair shaft formation, and hair growth cycles of the

mature follicle. Several IGF-binding proteins are expressed in the hair follicle, implicating them in hair follicle biology. However, the function of IGF-binding proteins in the hair follicle and skin has not been investigated extensively. IGFBP-3 controls the onset of the regression phase of the hair cycle but is expressed weakly in dermal papilla cells of anagen follicles, and its activity increases dramatically at the end of the growth phase (Schlake et al., 2004). Schlake (2005b) focused on IGFBP-5, which is also expressed in the hair follicle.

During embryogenesis and early postnatal development, 4 hair types are present: guard, awl, zigzag, and auchene (Dry, 1926). IGFBP-5-mediated fibroblast growth factor receptor-3b (FGFR2-IIIb) signals regulate the genetic program that controls the structure of the hair shaft medulla (Schlake, 2005a). Segmental IGFBP-5 expression is specifically associated with the bent structure of zigzag hair and is a central regulator of hair shaft differentiation and hair type determination (Schlake, 2005b). IGFBP-5 mediates the formation of the bent hair structure of zigzag hair. Sriwiriyanont et al. (2011) reported that overexpression of IGFBP-5 in human hair xenografts resulted in twisted hair shafts and unusual deposition of hair cuticles as a consequence of impairments in normal proliferation and differentiation. IGFBP-5 affects human hair shape, and lentiviral transduction can be used to analyze the function of genes in human hair morphogenesis (Sriwiriyanont et al., 2011).

Keratins and KAPs constitute a large, heterogeneous group of proteins that make up approximately 90% of wool fiber. *KAP6-1* is specifically expressed in hair follicles in sheep (McLaren et al., 1997). KAP6 expression begins relatively late in hair follicle differentiation, and the proportion of hair cortical cells that express it varies between follicles (Fratini et al., 1993). Based on the specificity of *KAP6-1*, the KAP6-1 promoter can be used to affect hair follicle-specific expression of other genes (Guo et al., 2009; Wang et al., 2010); it has been shown to drive downstream gene expression in hair follicles (Wang et al., 2008).

In this study, we demonstrated that our plasmid, in which a neomycin-resistance cassette and a red fluorescent protein are driven by a CMV promoter, is expressed specifically in hair follicles, thereby enhancing the efficiency of screening.

CONCLUSIONS

Goat IGFBP-5 expression plasmids were constructed and stably transfected into Cashmere goat fetal fibroblasts. DsRed was efficiently expressed in the cells. These data provide a method of constructing a hair follicle-specific expression vector for functional genes, and for obtaining stable transfection donor cells for nuclear transfer.

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