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Full Length Research Paper

Myogenic conversion of bladder fibroblasts by construction and expression of eukaryotic expression vector of myod1 gene

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Gene therapy of detrusor underactivity, by autologous cells transplantation, is limited by the number of primary myogenic. The purpose of this study was to investigate whether Myod1 could induce primary bladder fibroblasts to undergo myogenic conversion. Primary bladder fibroblasts from Sprague-Daley rats were cultured. The eukaryotic expression plasmid pEGFP-Myod1 carrying both a rat Myod1 cDNA and a green fluorescent protein reporter gene was constructed and identified. The cultured primary bladder fibroblasts were transfected by pEGFP-Myod1 with Lipofection 2000 reagent. The results showed that expression of Myod1 could cause myogenic differentiation of bladder fibroblasts. These findings support the possibility of an alternative approach to exploit the capacity of Myod1 to activate myogenesis in bladder fibroblasts *ex vivo* and to create a vast source of autologous myogenic cells for gene therapy of detrusor underactivty by cell transplantation.

Key words: Fibroblasts, Myod1, gene therapy, detrusor underactivity.

INTRODUCTION

Detrusor underactivity (DU) greatly remains a common and challenging clinical problem in elderly patients. DU is defined by the Standardization Subcommittee of the International Continence Society as a contraction of reduced strength and/or duration, resulting in prolonged bladder emptying and/or failure to achieve complete bladder emptying within a normal time span (Abrams et al., 2003). In fact, DU is very common in elderly women and men with lower urinary tract symptoms (Brierly et al., 2003). Urinary retention is generally caused by detrusor underactivity. Detrusor underactivity can lead to recurrent urinary tract infections and eventual kidney damage. Biopsy studies have established a strong relationship between urodynamic evidence of DU and structural changes. Detrusor muscle loss and collagen deposition represent two morphological features which are common and central to DU (Haferkamp and Elbadawi, 2004; Elbadawi et al., 1993). The *ex vivo* approach to gene therapy of DU is based on transplantation of autologous myogenic cells engineered to express a functional gene product. Cell transplantation is a promising strategy that offers the creation of new functional tissue to replace the lost or failing tissue (Southgate et al., 2003). However, the difficulty in obtaining sufficiently large quantities of suitable cells is clearly a limiting factor for the clinical application of cell transplantation (Bolland and Southgate, 2008). In recent years, Myod1 has been attracting the attention of researchers. It has been known that Myod1 is a member of the myogenic family of basic helix-loop-helix

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Abbreviations: DU, Detrusor underactivity; **RT,** reverse transcriptase; **PCR,** polymerase chain reaction; **MHC,** myosin heavy chain; **DMEM,** Dulbecco's modified Eagle's minimal essential medium; **FBS,** fetal bovine serum; **GFP,** green fluorescent protein; **BOO,** bladder outlet obstruction; **BPH,** benign prostatic hyperplasia.

transcription factors and functions as unique master genes that are able to prompt myogenesis in a variety of cells including fibroblasts (Weintraub et al., 1991). Transformation of bladder fibroblasts into skeletal myocytes in DU tissue could add to strength. Cell transplantation by Myod1 conversion of bladder fibroblasts *ex vivo* is an alternative strategy of bladder repair, because it combines the advantages of genetic and tissue engineering. This approach may provide an alternative source of autologous, transplantable, myo-genic cells by *ex vivo* genetic manipulation of bladder fibroblasts.

In this study, we constructed and identified a eukaryotic expression plasmid pEGFP-Myod1 and transfected it into the cultured primary bladder fibroblasts. The aim of our study was to investigate the problem of whether the phenomenon of myogenic conversion of primary bladder fibroblasts with Myod1 could be exploited in practical terms to provide an alternative source of myogenic cells for transplantation.

MATERIALS AND METHODS

Construction of eukaryotic expression vector of pEGFP-Myod1

Total RNA was taken from normal fresh Sprague-Daley rat muscle tissues. Extraction of total RNA was performed by following the instructions of the Trizol reagent (Invitrogen, USA). Total RNA was converted to cDNA by ABI high capacity cDNA archive kit (Applied Biosystems, CA) following the manufacturer's protocol. The Myod1 cDNA primers were designed according to the GeneBank (NM_176079). The sequence of upstream primer was: 5' GGACCCAGAACTGGGACATG-3' and the sequence of upstream primer was: 5'-CTGCAGCCAACCTCTCAGAG-3'. Both of them were constructed by Invitrogen Life Technologies. The RT products were then used as template for PCR amplification. The products were retrieved by gel extraction kit (Omega, USA). The PCR products were directly cloned into pMD18-T simple vector (Takara, Japan) and the $CaCl_2$ method was used to convert *Escherichia coli*. The pEGFP vector (Clontech, CA) was digested by restriction endonuclease enzyme NotI and EcoRI (NEB, USA) and the 3.4 kb fragment was retrieved by gel extraction kit (Omega, USA). Myod1 DNA was digested by BamHI and EcoRI and the 1 kb fragment was retrieved by the same way, which was then cloned into the vector of digested pEGFP. The reaction system consisted of 2 µl target genes, 2 µl (5×) buffer, 2 µl pEGFP, 1 µl T4 DNA ligase (NEB, USA), 4 µl sterilized water, in which the target genes and DNA plamids were ligated. After overnight storage at 4°C, 5 µl ligation products was taken for the conversion of *E. coli* DH5α treated by cool $CaCl_2$. After overnight culture at LB medium containing ampicillin, single colony was selected at random and cultured with shaking. The DNA plasmids were then extracted by using plasmid giga kit (Omega, USA). The recombinant plasmid was named as pEGFP-Myod1. The target DNA was sequenced by Invitrogen Life Technologies.

Primary bladder fibroblasts culture

Primary bladder fibroblasts were derived from newborn Sprague-Daley rats. The bladders were harvested, minced and added to dissociation solution containing 0.5 mg/ml Collagenase (GIBCO, CA). The cell solution was filtered through a 40 µm cell strainer and spun at 1,000 rpm for 5 min. The supernatant was aspirated and cells were resuspended in DMEM-F/12 medium containing 10% FBS and 1% Penicillin/Streptomycin (GIBCO, CA). Cells were maintained at 37°C and 5% CO_2 in a saturated humidified incubator (Sanyo, Japan). The cell number was determined and cells were plated at a density of 25,000 cells/cm^2 in 24-well plates (Corning, USA) for biochemical and immunocytochemical studies. Cell labeling studies was used to evaluate the expression of fibroblast markers.

Transient transfection

Lipofectamine 2000 transfection reagent (Invitrogen, USA) was used for the transfection of primary bladder fibroblasts. According to the size of 24 well plate, the cells were transfected with Lipofectamine 2000 transfection reagent: DNA amounts of 3 µl:1 µg in each well. 97 µl serum-free medium and 3 µl Lipofectamine 2000 transfection reagent and 1µg plasmid were added in a small sterile tube, in turn and with rocking gently to mix and then incubated for 20 min at room temperature. Add the complex mixture to each well containing cells. Incubate cells at 37°C in a 5% CO_2 incubator and then replace the medium with serum-containing medium 5 h after transfection. Cells were harvested 48 h after transfection. Transfection efficiency was determined 24 h after transfection by the extent of the expression of GFP reporter gene with a fluorescent inverted microscope.

Immunocytochemistry

Cultured cells were stained using the monoclonal antibodies for vimentin, desmin or fast myosin heavy chain (MHC) (Santa Cruz, CA). Briefly, cells were fixed with 4% paraformaldehyde, washed in PBS. Endogenous peroxidase was quenched with 3% H_2O_2. Cells were incubated with the primary antibodies in PBS with 1% BSA. This was followed by washes, incubations with an appropriate biotinylated secondary antibody, DAB development. For immunofluorescence analysis, the monoclonal antibody for fast MHC was visualized with CY3-tagged goat anti-mouse IgG. Cultures were then washed, mounted in 75% glycerol/PBS (pH= 7.5) and examined under an Eclipse E400 fluorescence microscope (Nikon, Japan).

RESULTS

Sequencing of rat Myod1 gene

Clones were send to sequence and the results indicated that plasmid pEGFP-Myod1 was correctly constructed (Figure 1). The sequencing of the rat Myod1 gene showed that it was 957 bp in open reading frame, which was fully in consistency with that of Genebank (NM_176079).

Characterization of primary bladder fibroblasts

Cell labeling studies established that essentially all of our cultured cells expressed fibroblast markers, with no apparent contamination by other cells. Theoretically, fibroblasts were stained positive for vimentin (Figure 2) and stained negative for desmin. In the study of immunocytochemistry, the results showed that there were

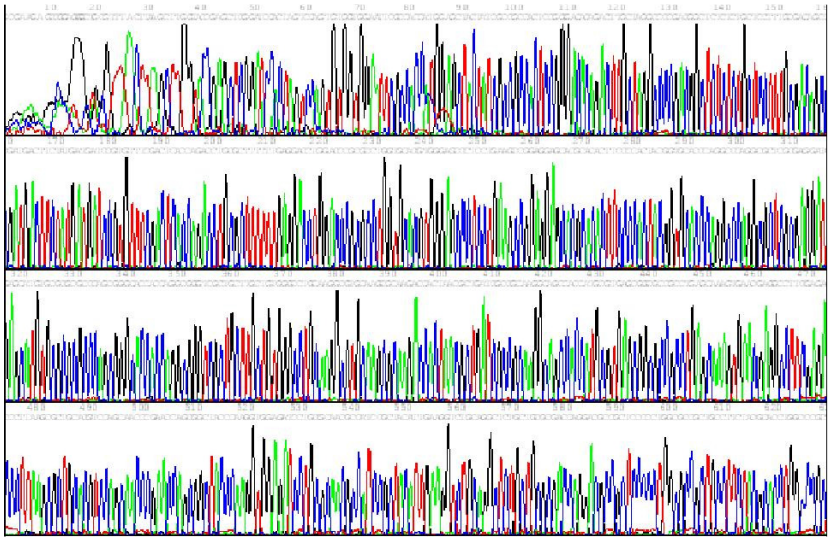

Figure 1. DNA sequencing of pEGFP-Myod1.

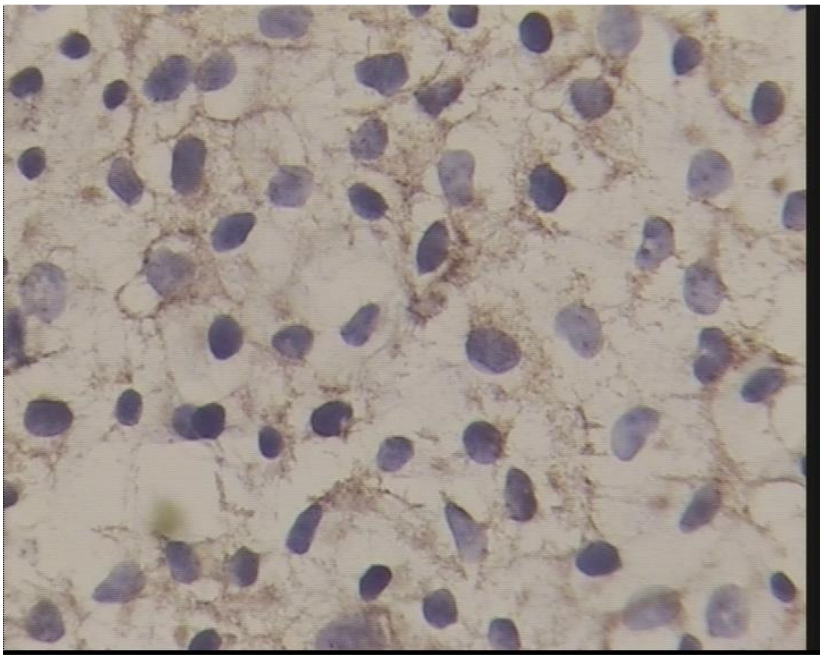

Figure 2. Immunostaining for vimentin (×250). The brown color stains identified positive cells.

positive expression of vimentin and negative expression of desmin, suggesting that our cultured cells were fibroblasts.

Myogenic conversion of primary bladder fibroblasts

Primary bladder fibroblasts were transfected with plasmid pEGFP-Myod1 encoding the Myod1 gene and the GFP reporter gene. Twenty-four hours after transfection, expression of the GFP reporter gene was identified using fluorescent microscopy, indicating efficient transfection of the GFP transgene in 95% of the fibroblasts (Figure 3). In order to assess the differentiation of the transfected cells, immunofluorescence staining against fast MHC was used. Theoretically, myotubes were stained positive for fast MHC, indicating myogenic differentiation. Forty-eight hours (48 h) after transfection, the cells were stained positive for fast MHC (Figure 4). These myogenic changes were undetectable in control fibroblasts.

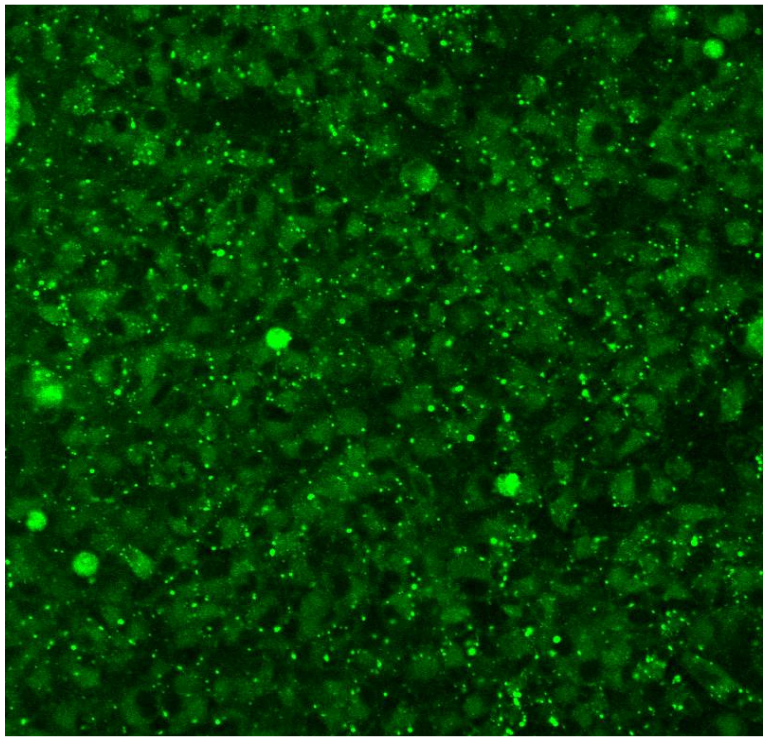

Figure 3. Immunofluorescence staining for GFP (×200). The green color stains identified positive cells.

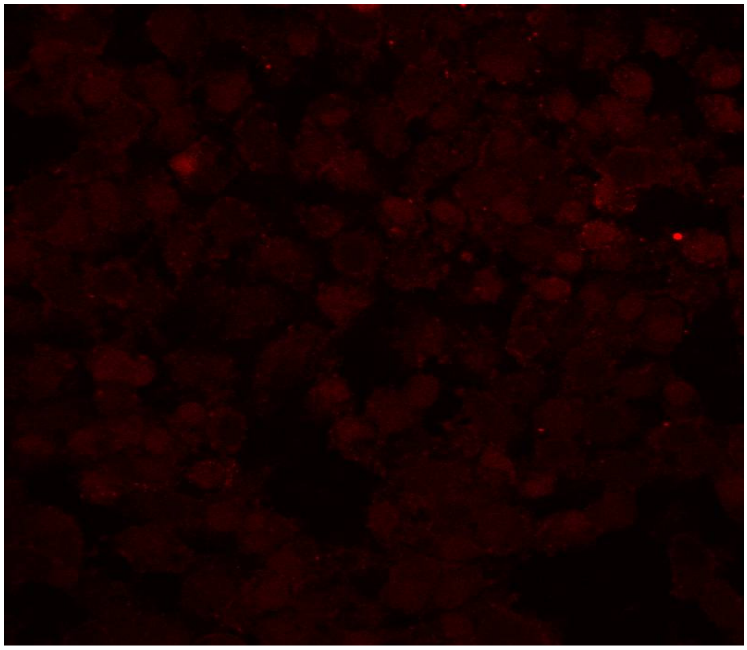

Figure 4. Immunofluorescence staining for fast MHC (×400). The red color stains identified positive cells.

DISCUSSION

In this study, we have shown for the first time that bladder fibroblasts can be converted to myogenesis by forced expression of Myod1. The data reported in this paper suggest that Myod1 conversion of bladder fibroblasts *ex*

vivo may provide an alternative therapeutic source of transplantable myogenic cells for DU.

DU is very common in elderly and patients, particularly in those who are frail (Brierly et al., 2003). It develops in some older men with bladder outlet obstruction (BOO) due to benign prostatic hyperplasia (BPH) (Griffiths et al., 2002). DU related urinary retention could further accelerate the progression of this condition by bladder distension, which is similar to those mechanisms occurring in BOO (Taylor et al., 2006). The management of DU represents especially unsatisfactory, because the method of using bethanechol to enhance detrusor contractility is ineffective (Harada et al., 2010). The effectiveness of surgical approaches to BPH is placed into question by the presence of DU (Griffiths et al., 2002). Detrusor muscle loss and collagen deposition represent two morphological features which are common and central to the condition of DU (Taylor et al., 2006; Haferkamp and Elbadawi, 2004). The development of these structural changes correlates with symptom severity (Siroky, 2004; Mirone et al., 2004). Moreover, these structural changes may in fact precede the development of urodynamic evident of DU and may herald the development of DU (Elbadawi et al., 1997; Mastropietro et al., 2001).

The transformation of bladder fibroblasts of DU into muscle may improve bladder function. It has been known that the forced expression of members of the myogenic family of basic helix-loop-helix transcription factors in cultured non-muscle cells could initiate the process of myogenesis (Weintraub et al., 1991). This process is directly related to the lineage relationship with myoblasts and possible development tendency of embryonic cells towards myogenesis (Bergstrom and Tapscott, 2001). The genetic manipulation of fetal fibroblasts by adenoviral vector encoding Myod1 could provide a source of transplantable myogenic cells in a mouse model of skeletal injury (Fujii et al., 2006). The findings of our study indicate that the eukaryotic expression vector of Myod1 gene converted fibroblasts may provide a feasible alternative strategy for detrusor repair by cell transplantation and ex vivo gene therapy.

Cellular transplantation is an innovative approach to repair the injured bladder smooth muscle cells (Southgate et al., 2003). Recent interest has focused on autologous cells that would avoid rejection of transplanted tissues (Memon et al., 2005). After transplantation into the bladder, myogenic skeletal muscle cells might undergo transdifferentiation into functioning bladder smooth muscle cells (Southgate et al., 2003). However, there are two major barriers for the clinical application of autologous skeletal myoblasts in elderly patients. One is the low recovery of satellite cells from muscle biopsies (Mouly et al., 2005); the other is the reduced capacity to create functional myofibers (Renault et al., 2000). Therefore, another autologous cell source might be needed. An alternative strategy is *ex vivo* cellular transformation, which is *ex vivo* genetic manipulation of bladder fibroblasts by vector encoding Myod1 and creation of sources of transplantable myogenic cells. This approach combines the advances in genetic and tissue engineering. One major advantage is that fibroblasts are autologous, abundant, easily expandable and simple to harvest from clinically assessable sites. The other advantage is that transient forced expression of the Myod1 transgene activates endogenous Myod1 and irreversibly assigns cells to myogenesis. This study implies that *ex vivo* cellular transformation is an attractive and better alternative strategy.

In conclusion, we constructed and identified a eukaryotic expression plasmid pEGFP-Myod1 and transfected it into primary bladder fibroblasts. It was observed for the first time that bladder fibroblasts can be converted to myogenesis by forced expression of Myod1. The recombinant plasmid pEGFP-Myod1 may serve as a helpful tool for the further study of gene therapy of DU. We showed that it is possible to exploit the unique capacity of Myod1 to activate myogenesis in bladder fibroblasts to create a virtually infinite source of autologous myogenic cells for DU therapy.

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