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Original Article

A gene expression profile analysis of the differentiation of muscle-derived stem cells into smooth muscle cells from sheep

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Abstract: Objective: To analyze gene expression profiles analysis during the differentiation of sheep muscle-derived stem cells (MDSCs) into smooth muscle cells (SMCs) in sheep. Methods: A modified preplate technique was employed to isolation of the MDSCs from sheep. The MDSCs were subjected to flow cytometry analysis targeting CD44, CD31, CD45, CD14, and CD49f and were treated with TGF-β1 at a concentration of 10 ng/ml for ten days. The expression levels of smooth muscle α-actin (α-SMA) and calponin after treatment with TGF-β1 were determined by western blotting and immunofluorescence staining. A microarray analysis was performed to screen for differentially expressed genes (DEGs) during MDSC differentiation using total RNA extracted from MDSCs and SMCs generated from MDSCs. Molecule Annotation System (MAS) 3.0, which employs KEGG (Kyoto Encyclopedia of Genes and Genomes) and GO (Gene Ontology) Consortium annotations, was used to identify global biological trends in the gene expression data. Results: The expression levels of the SMC-specific contractile proteins α-SMA and calponin were dramatically increased after treatment with TGF-β1. Immunofluorescece staining showed that the TGF-β1-treated MDSCs were positive for α-SMA. We identified 486 genes that were differentially expressed between the MDSCs and TGF-β1-treated MDSCs. 260 of which were up-regulated and 226 of which were down-regulated. Twenty-one genes exhibited a greater than ten-fold change, 13 of which were up-regulated and 8 of which were down-regulated. TGF-β1 treatment up-regulated both the SMAD and MAPK signaling pathways during the differentiation of these sheep cells. The PPAR and Wnt signaling pathways were also found to be involved in the differentiation process. Conclusions: TGF-β1 can successfully induce the differentiation of sheep MDSCs into SMCs. For the first time, we analyzed the gene expression profiles associated with this differentiation process, and the results showed that both the SMAD and MAPK signaling pathways are involved. This study indicated that multiple signaling networks coordinate the development and differentiation of MDSCs into SMCs.

Keywords: Muscle-derived stem cell, smooth muscle cell, transforming growth factor-β, microarray analysis, sheep

Introduction

Pelvic organ prolapse, specifically vaginal prolapse, is highly prevalent and may occur in up to 50% of parous women [1, 2]. The surgical cure rates vary, and recurrences are common after primary ungrafted methods of vaginal repair are applied. Grafts performed for vaginal repair following prolapse have been proven to be effective with level A evidence. However, complications may occur in graft transplantations. One of the main complications is exposure of the graft. To reduce the rate of exposure, grafts carrying stem cells to regenerate vaginal tissue have been tested. Our assumption is that the stem cells could differentiate into smooth muscle and increase the thickness of vaginal smooth muscle in order to reduce exposure of graft.

Muscle biopsies allow autografts of muscle-derived stem cells (MDSCs) to be isolated.

MDSCs have been characterized in rodents and isolated from human muscle [3, 4]. These cells differ from the myogenically committed satellite cells studied in association with stress urinary incontinence and vaginal repair [5]. Remedial strategies employ regenerative medicine to repair the vaginal or pelvic floor and show great promise for restoring function and improving the quality of life.

Among non-primate animals, sheep exhibit the most similar vaginal anatomy to human [6]. In a previous study, we grew MDSCs on small intestinal submucosa (SIS) to generate smooth muscle cells (SMCs) in vitro, and we subsequently implanted them into a sheep vaginal defect model (unpublished). There are available studies on the differentiation of MDSCs into SMCs in rats and human. In Park's research, TGF-1 induces the increased expression of SMC-like ion channels and the differentiation of Mesenchymal stem cells into smooth muscle [7]. And the Wnt5a_Ror2 pathway is also associated with determination of the differentiation fate of mesenchymal stem cells [8]. However, the gene expression profiles and molecular mechanisms controlling the differentiation of MDSCs in sheep are still poorly understood. In this study, we investigated the changes in gene expressions that occur during the differentiation of MDSCs into SMCs.

Method

Cell isolation and culture

We employed a modified preplate technique to isolate sheep MDSCs based on the protocol of Burhan Gharaibeh et al. [9]. To further enrich the MDSCs, we performed the following series of steps to eliminate any fibroblast-like cells from the culture. Remove the tissue culture medium and wash the flask with 3 ml DPBS. Gently apply 2 ml of 0.25% (wt/vol) trypsin solution to the flask. Incubate for approximately 1 min at 37°C. Follow that by gently adding 2 ml of PM to stop the enzymatic process. Transfer the resulting solution into a 15-ml conical centrifuge tube and centrifuge at 930 g at 4°C for 5 mins to pellet the cells that detached during the trypsinization treatment. Decant the supernatant and resuspend the pellet in 5 ml of PM, then plate in a fresh collagen-coated flask. Allow the cells to adhere to the flask for 30 min at 37°C. Remove the supernatant containing any nonadherent cells and 'replate' into a new collagen-coated flask. These MDSCs have been well characterized on basis of surface markers. They are positive for CD44 and CD49f, but negative for CD31, CD14 and CD45 (AbDSerotec, Kidlington, UK). The isolated MDSCs were cultured in DMEM supplemented with 10% FBS, 10% horse serum (Invitrogen), 0.5% Chick embryo extract (AccurateChemica) and 100 U/ml penicillin/streptomycin that had been steriled via filtration through a 0.22 mm filter. The cells were maintained in a humidified incubator at 37°C with 5% CO_2. MDSCs up to third passage were used in our experiments. The study was approved by the ethics committee of Fu Wai hospital.

Flow cytometry

To confirm that the MDSCs maintained their phenotype following expansion in culture, the cells were subjected to flow cytometry analysis. The cells were detached via trypsin treatment, then centrifuged and washed with PBS. The samples were incubated with primary antibodies conjugated to FITC (targeting CD44, CD31, CD45, CD14 and CD49f) for 30 min at 4°C.

TGF-β treatment

The MDSCs were treated with TGF-β1 (Merck Millipore, Darmstadt, Germany) at a concentration of 10 ng/ml. Previous studies have shown that TGF-β1 at concentrations of 5 and 20 ng/ml induces similar expression levels of smooth muscle α-actin (α-SMA) and collagen I expression in mesenchymal stem cells [10]. For long-term culture, TGF-β1 was supplemented when the cultured medium was changed, which was carried out approximately every 2-3 days.

Immunofluorescence staining

TGF-β1-treated MDSCs were cultured on sterile cover slips in 24-well plates at a density of 10^3 cells/cm^2. A primary antibody to detect α-SMA was applied at a dilution of 1:100. The slides were analyzed using a Leica microscope.

Western blot analysis

After the cells had been treated with TGF-β1 for 10 days, they were washed twice with PBS and then subjected to protein extraction using RIPA buffer. Antibodies against α-SMA (Sigma) and calponin (ABBIOTECH) were used to detect SMC-specific protein expression. Vaginal sm-

Table 1. Primer sequences used for qRT-PCR

Gene name	Forward primer (5' to 3')	Reverse primer (5' to 3')
GAPDH	ACACTGAGGACCAGGTTGTCT	GGGGGTCCAAGGACCTTACT
CD73	GGTGATCCTCCCAAGCTTCC	CCACATTGATATCTTGGTCACAGAG
CD44	AGAACGAACCCCGAAGACATC	GGTACACAGTTGGGAGGTGC
Calponin	GGAAAGAGCAAGAAGCCCCT	ATCAGCCAAGAAGTGGGTGC
α-SMA	CTAATGTGCGAGGAGGAGGAC	GAGGACGTCCCACGATAGAC
Mf5	CCAACCCTAACCAGAGGCTG	ACTGCTGCTCTTTCTGGACC
WNT5	AAGACGGGCATCAAGGAGTG	CGGGGAGAAGGGGATAGTCA
WIF1	AACAAGATGGAGTGGCAGCA	GGACATTGACGGTTGGGTCT
ATF3	AGTCACCAAAGCCGAGTAGC	TGCCTTCAGCTCAGCATTCA
SMAD	ACTCCAGGACGCTGTTGGTA	GGAGGGGGAGACTCACGAA
WISP1	GGCCACACATCAAGAAGGGA	TCGGGACACTGGAAGGAG

ooth muscle cells (VSMCs) were employed as a positive control. The method of isolating VSMCs was from the reference [11].

RNA extraction and microarray analysis

Total RNA was extracted using the TRIzol reagent and was further purified using the Qiagen RNeasy Mini Kit according to the manufacturers' instructions. The RNA concentrations were quantified with an ultraviolet Spectrophotometer (NanoDrop Technologies, ND-1000), and the quality of the RNA was assessed via formaldehyde agarose gel electrophoresis. An aliquot of 200 ng of total RNA was used to synthesize double-stranded cDNA, and the modified nucleotide, biotin-UTP, was incorporated into the antisense RNA synthesized by the MessageAmp™ Premier RNA Amplification Kit (Life Technologies Corporation). Then Fragment the biotinylated aRNA to strands of 35-200 bases in length according to the Affymetrix' protocols prior to hybridization. After measuring the concentration and verified the fragment length, the biotinylated aRNAs were hybridized to Ovine Gene 1.1 ST Array Strip (AffymetrixInc, Santa Clara, CA) containing 22047 transcripts. Hybridization was performed at 45°C with rotation for 16 h with constant rotation at the speed of 60 rpm in a hybridization oven 640. After washing and automatic staining on an Affymetrix fluidics station 450, the GeneChip arrays were scanned on AffymetrixGeneChip Scanner 3000 7G. The scanned images were first assessed via visual inspection, and then analyzed to generate CEL files using the default settings of Affymetrix® GeneChip® Command Console® 3.2 (AGCC) software. The raw data were normalized and summarized with Affymetrix Microarray Suite 5.0 (MAS5) and with the Robust Multi-array Average (RMA) algorithm (Irizarry et al., 2003). During the experiments, we used controls provided by Affymetrix according to their protocols. These quality controls ensured the reliability of the results.

Real-time PCR validation

Total RNA was treated with DNase I to eliminate the traces of genomic DNA. Then 2 μg of Total RNA was employed to synthesize the first strand of cDNA in a 20 μl reverse transcription (RT) reaction. Next 2 μl of the RT product were subjected to PCR amplification using the LightCycler PCR system (Roche Molecular Biochemicals, Mannheim, Germany) with SYBR-Green I monitoring method. The relative expression ratio of a target gene was calculated based onthe amplification efficiencies (E) and the crossing point (CP) deviation of an unknown sample versus a control, and is expressed in comparison to a reference gene:

$$\text{Ration} = \frac{\triangle CP_{target}\ (\text{control} - \text{sample})}{\triangle CP_{ref}\ (\text{control} - \text{sample})}$$

The glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene was used as the reference gene. The amplification efficiencies of the different target genes were calculated independently according to the statistical delimitation of the beginning of exponential behavior observed in the real-time PCR kinetics. The target genes included four genes which were associated with the characteristics of stem cells and smooth muscle cells and six genes associated with TGF-β1-relatedsignaling pathways. The primer sequences for these genes are listed in **Table 1**. All real-time PCR experiments were carried out with three biological replicates, and three technical replicates were performed for every sample.

Gene ontology and KEGG pathway analysis

The scanned signals were extracted with AGCC software (Affymetrix). A gene was recognized as differentially expressed when the change in

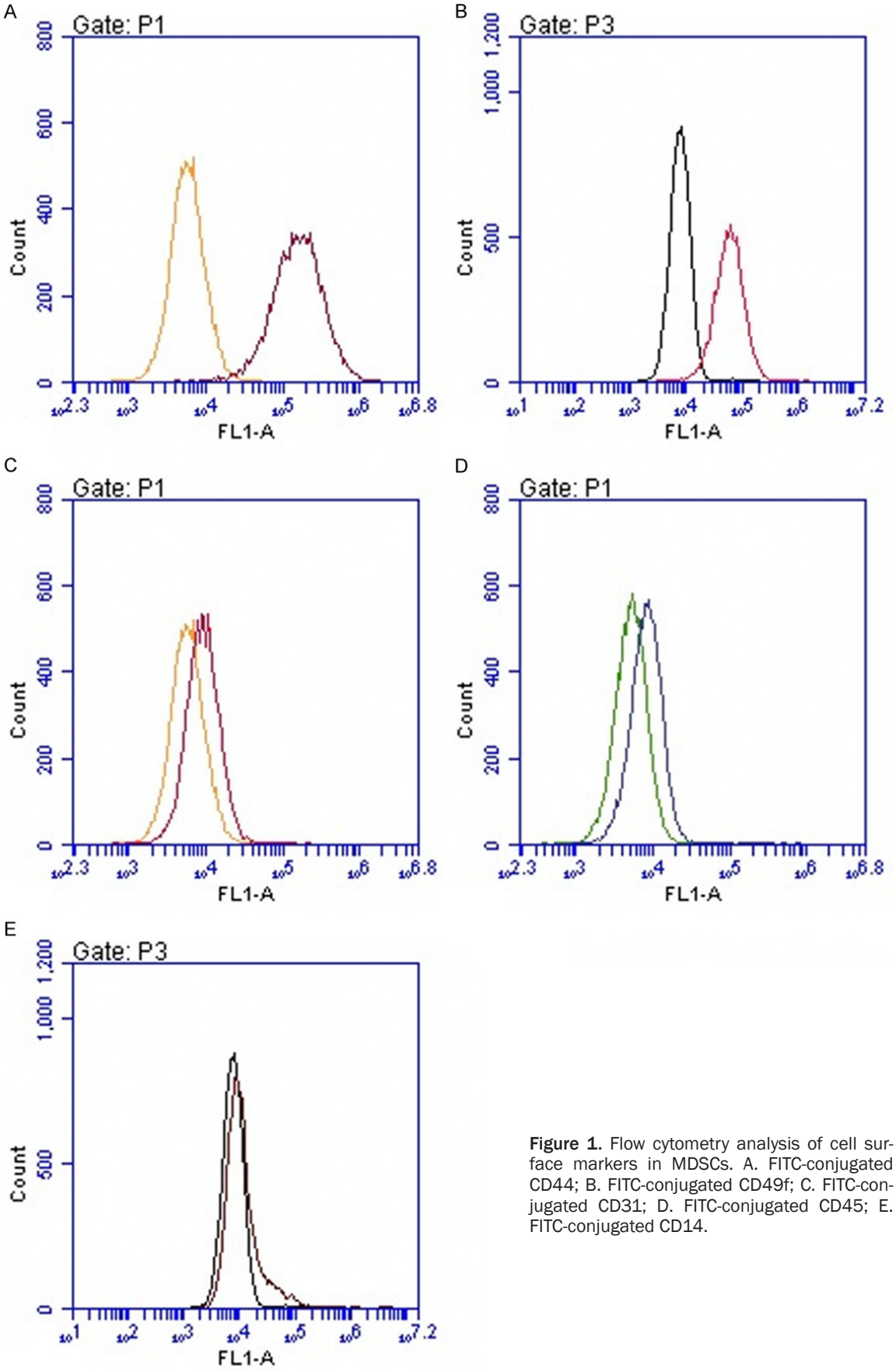

Figure 1. Flow cytometry analysis of cell surface markers in MDSCs. A. FITC-conjugated CD44; B. FITC-conjugated CD49f; C. FITC-conjugated CD31; D. FITC-conjugated CD45; E. FITC-conjugated CD14.

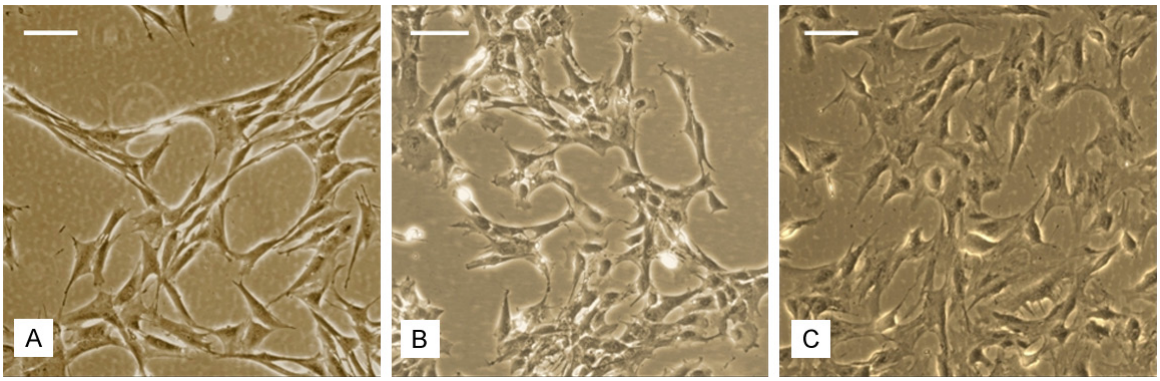

Figure 2. Effect of TGF-β1 on MDSC morphology. A. MDSC before differentiation (×100); B. MDSC after 2 days in differentiation medium (×100); C. MDSC after 10 days in differentiation medium (×100).

its signal was greater than 2.0-fold. A dataset composed of the differentially expressed genes was entered into Molecule Annotation System (MAS) 3.0 software (CapitalBio, http://bioinfo.capitalbio.com/mas3/), which is a website-based program that was designed for microarray data analysis and molecule annotation. MAS uses KEGG and GO Consortium annotations to identify global biological trends in gene expression data. MAS calculates the total number of genes within a KEGG pathway or a GO term showing changes, together with a statistical score (*p*-values and q-values, or the false discovery rate), providing a comprehensive view of the differences in gene expression differentiation associated with that particular pathway or term.

Result

Identification of MDSCs

Flow cytometry analysis showed that the MDSCs were positive for CD44 (99.5%) and CD49f (92.4%) but negative for CD31 (9.1%), CD14 (10.2%) and CD45 (2.4%) (**Figure 1**).

TGF-β1-induced differentiation of MDSCs into SMCs

We observed morphological changes in the MDSCs after 2 days of culture in the TGF-β1-containing medium (**Figure 2**). TGF-β1 caused MDSCs to adopt a spindle-shaped functional SMC morphology. This cell morphology was maintained as the cells grew. To determine whether the MDSCs could be differentiated into SMCs using TGF-β1, we exposed them to TGF-β1 for 10 days. As shown in **Figure 3**, the expression levels of the SMC-specific contractile proteins α-SMA and calponin were dramatically increased by treatment with TGF-β1. The immunofluorescence staining results showed that the TGF-β1-treated MDSCs were positive for α-SMA (**Figure 4**).

Expression pattern

In a comparative analysis, we applied a two-class unpaired method in the Significant Analysis of Microarray software (SAM, version 3.02) to identify significantly differentially expressed genes between untreated MDSC group and TGF-β-treated MDSC groups. Genes showing more than a two-fold change in expression were considered to be differentially regulated. The results showed that 486 genes were differentially expressed between the MDSCs and TGF-β1-treated MDSCs, 260 of which were up-regulated and 226 of which were down-regulated. Twenty-one genes exhibited a greater than ten-folds change, 13 of which were up-regulated and 8 of which were down-regulated.

To identify genes undergoing significant changes in expression levels during MDSC differentiation, the expression levels often genes were investigated. CD44 and CD73 were found to down-regulated, whereas Myf5, Calponin and α-SMA were up-regulated. This result indicated that the stem cell characteristics were declining and the SMC characteristics were on the rise during differentiation. Genes related to the SMAD, Wnt, mitogen-activated protein kinase (MAPK) and peroxisome proliferator-activated receptors (PPAR) signaling pathways were also up-regulated (**Table 1**). The tendencies revealed by the gene microarray and RT-PCR analysis were the same.

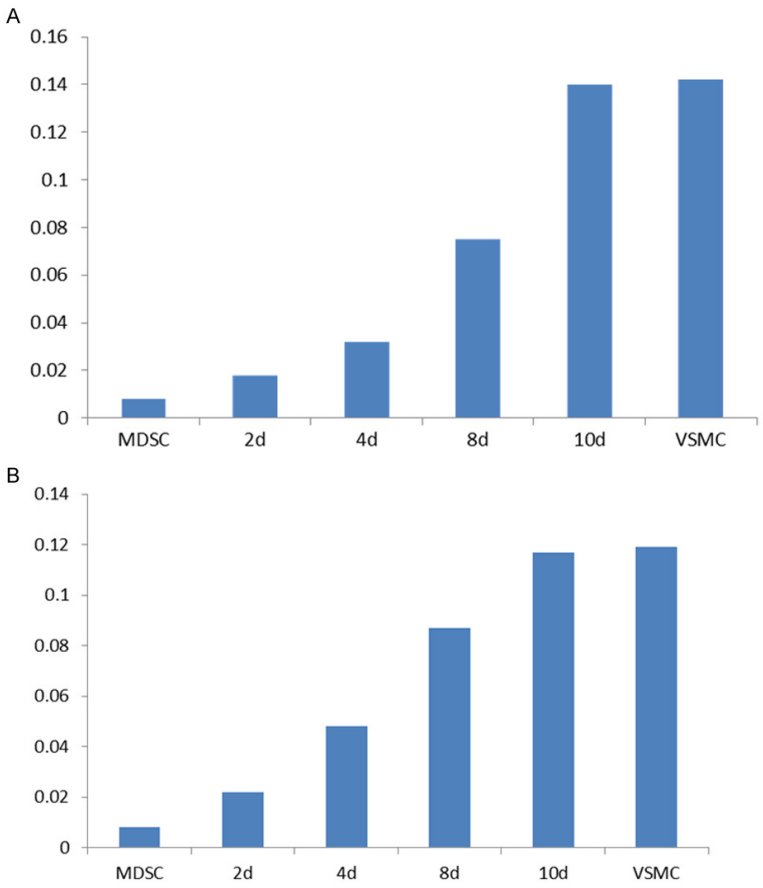

Figure 3. Expression of SMC-specific proteins (α-SMA and calponin) detected by western blotting. A. Expression of α-SMA in MDSCs before and after differentiation. B. Expression of Calponin in MDSCs before and after differentiation Y axis indicates absorbance.

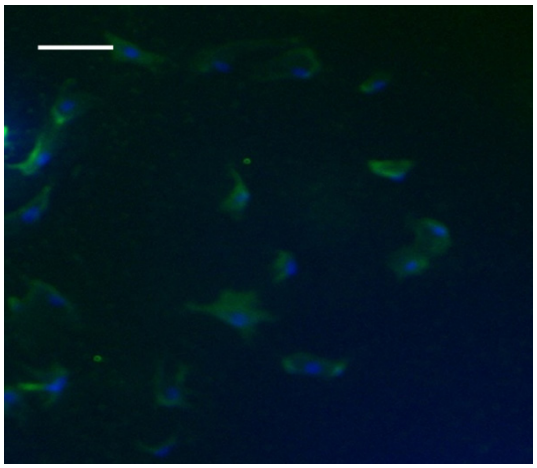

Figure 4. Immunofluorescence of MDSCs after 10 days of TGF-β1 Treatment. Expression of α-SMA in sheep MDSCs after 10 days of TGF-β1 treatment Scale bars shown in each figure represent 100 μm (×200).

GO annotation

To obtain a comprehensive picture of global gene expression, we use GO annotations to determin the groups of functionally relevant genes. Three main classes of processes were distinguished: (1) Biological processes, (2) Cellular components and (3) Molecular functions (**Figure 5**). We defined the significantly regulated processes as those showing a fold change of at least 2.0.

There were 516 biological processes identified. The most significant processes were GO:0042127, regulation of cell proliferation; GO:0048731, system development; GO:0048583, regulation of response to stimulus; GO:0022603, regulation of anatomical structure morphogenesis; GO:0048518, positive regulation of biological process.

Forty-one statistically significant molecular function-related processes were identified. The most significant processes were GO:0070851, growth factor receptor binding; GO:0005126, cytokine receptor binding; GO:0005114, transforming growth factor beta receptor binding; GO:0019199, transmembrane receptor protein kinase activity; GO:0008083, growth factor activity.

In the cellular component category, there were 28 statistically significant processes identified. The most significant processes were GO:0005576, extracellular region; GO:0009986, cell surface; GO:0031012, extracellular matrix; GO:0005615, extracellular space; GO:0071944, cell periphery.

KEGG pathway

A KEGG pathway analysis was performed to illustrate all of the available pathways that included differentially expressed genes. We

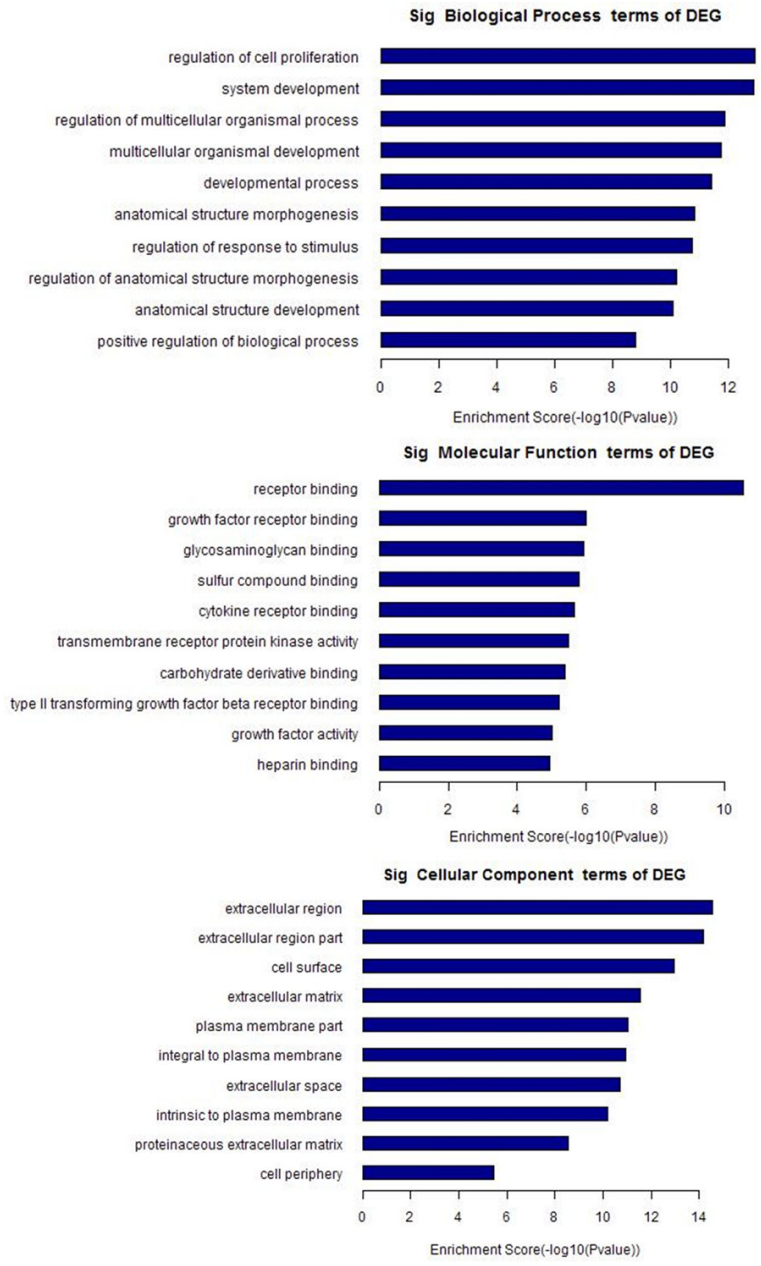

Figure 5. GO functional enrichment analysis of DEGs during differntiation.

defined the significantly regulated pathways as those with both a *p*-value and a q-value of less than 0.05. The results showed that gene expression was significantly enriched in the following categories: Cytokine-cytokine receptor interaction, PPAR signaling pathway, Regulation of actin cytoskeleton, Chemokine signaling pathway, TGF-β signaling pathway and the MAPK signaling pathway ($P<0.05$).

Discussion

MDSCs are promising potential gynecological therapeutic agents. In this context, the area of research receiving the most interest is the application of MDSCs to treat with pelvic floor dysfunction diseases. Much research has also focused on Stress Urinary Incontinence and pelvic organ prolapse [12-15]. Since 2008, two peer-reviewed articles have been published using MDSCs to treat Anal Incontinence [16, 17]. In previous study, We used two different methods to differentiate muscle-derived stem cells into smooth muscle cells in vitro and we implantated these cells into a sheep vaginal defect model (unpublished). In the present study, we used TGF-β1 to induce MDSCs to differentiation.

TGF-β1 is a multifunctional growth factor that regulates a broad range of biological processes, including cell proliferation, cell survival, cell differentiation, cell migration and production of the extracellular matrix (ECM) [18, 19]. Some researches have focused on the differentiation to obtain vascular smooth muscle cells [20, 21]. Combined with its stimulatory effect on MSC proliferation, TGF-β1 signaling therefore allows mesenchymal stem cells (MSCs) to expand [22]. The TGF-β1-induced modulation of MSC differentiation involves different lineages, including SMCs. TGF-β1 signaling contributes to the development of SMCs from embryonic stem cells [23]. The first evidence of the role of TGF-β1 in the differentiation of MSCs into SMCs was reported by Kinner research who found that TGF-β1 significantly increased α-SMA (an early marker of SMC differentiation) expression level [24]. Subsequently, Wang found that TGF-β1 at 5 and 20 ng/ml induced similar levels of α-SMA and collagen I expression in MSCs. Gong et al. showed that a TGF-β1 concentration of 0.1-10 ng/ml TGF-β1 inhibited human MSC proliferation but in creased the expression of calponin

(a late-stage SMC differentiation marker) expression in a dose-dependent manner [25], indicating that TGF-β1 not only initiates SMC differentiation but also promotes further differentiation. In the present study, we used a dose of 10 ng/ml. The increased expression of α-SMA and calponin increasing indicated that TGF-β1-treatedsheep MDSCs successfully differentiated into smooth muscle cells. In present study, we noticed that there are a small amount of α-SMA and calponin expressing in MDSCs group. We thought that there are small amount smooth muscle cells mixing into MDSC during the isolation process. We will modify the purifying process in the future.

TGF-β1 initiates signaling when its extracellular domain binds to two types (type I and type II) of transmembrane serine-threonine kinases receptor, which form a complex at the cell surface. The binding of a ligand binding to this complex induces a conformational change results in the phosphorylation and activation of the type I receptors by the type II receptors. The activated receptors subsequently phosphorylate the effectors Smad2/Smad3. Phosphorylated Smad2/Smad3 then form complexes with the common Smad (Smad4), which then translocate into the nucleus, where they interact with other transcription factors at the promoter regions at DNA sequence-specific binding sites for ATF2 (activating transcription factor-2) and SBE (Smad binding element) to regulate gene expression. The heteromeric Smad complex in the nucleus also interacts with various transcriptional co-activators or co-repressors resulting in the activation or the repression of downstream target genes [26]. Shi's research found that it might be that TGF-β/Smad3 induce transcription of genes associated with differentiation (NGF and Wnt-11) as well as de-differentiation, but the balance of their combined effect favors de-differentiation [27]. In our research, our result showed the effect favor differentiation. The reason maybe was Shi's research focused on vascular smooth muscle cell but our research focused on vaginal smooth muscle cell. And what we use in our experiment was TGF-β1.

TGF-β1 also induces non-SMAD signaling pathways through activation of the MAPK pathway [28]. In the present study, we found that SMAD was up-regulated and that genes in MAPK pathway were also significantly enriched. These findings indicate that TGF-β1 induces both the SMAD and MAPK signaling pathways during differentiation in sheep.

Although the mechanisms that regulate the differentiation of MDSCs into SMCs are beginning to be understood, many aspects of the specific intracellular signaling network involved in TGF-β1-induced MDSCs differentiation remain unknown. In a recent study, Xin et al. found that the Wnt pathway was associated with the differentiation of mesenchymal stem cells [29]. Additionally, a study by Lien showed that PPAR pathway was involved in TGF-β1-induced differentiation during differentiation into SMC [30]. In the present study, genes in the PPAR and Wnt signaling pathway were significantly up-regulated indicating that the mechanisms regulating the differentiation MDSCs into SMC are complicated and involved multiple signaling pathways. In future, we are going to compare the signaling pathway of differentiation when we use the two different methods and measure protein expression of downstream molecules in both pathways.

In conclusion, this study represents the first gene expression profile analysis of the TGF-β1-induced differentiation of sheep MDSCs into SMCs. The results showed that both the SMAD and MAPK signaling pathways participate in the differentiation process. Thus, multiple signaling networks must coordinate the development and differentiation of MDSCs into SMCs. It provides new insights into this differentiation mechanism. A study examining the DNA methylation of the genes showing differential expression during differentiation is planned.

Disclosure of conflict of interest

None.

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