Cytokine CCL7-mediated activation of mesenchymal stem cells to promote urinary continence via periurethral fibroblasts mechanism

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Research Article
Abstract

Stress urine incontinence (SUI) is common in middle-aged and older people, and there are no effective treatments. In our investigation, MSC secretion activated periurethral fibroblasts. MSC secretion concentrate improves stress urinary incontinence in animal models. Our work indicated that CCL7 recruits activated MSC cells. This study compared the omics expression of associated secretions after CCL7 was added to activate mesenchymal stem cells and the molecular regulatory mechanisms involved.

Periurethral fibroblasts were immortalised from patients with urine incontinence and anterior pelvic prolapse. Proteomic analysis was used to examine the composition of conditioned media obtained from bone marrow stromal cells and to study the link between fibroblast proliferation and migration and, eventually, signal route incurred changes. We identified the most plausible PI3k/AKT signal transduction route for activating periurethral fibroblasts generated by CCL7 and MSC secretions.

CCL7+MSC-CM promoted collagen production, proliferation, and migration of periurethral fibroblasts better than MSC-CM. PI3k/AKT-related pathways linked with increased fibroblast proliferation and migration were activated.

After CCL7 intervention, MSC-CM activated periurethral fibroblasts through PI3k/AKT. EFNA1 may play a critical role in the proliferation of periurethral fibroblasts, contributing to urinary continence and architecture.

Introduction

Stress urinary incontinence (SUI) is a very common medical condition in adult women occurring during coughing, sneezing, lifting heavy weights, etc. SUI is defined as involuntary urine leakage when the abdominal pressure increases, usually related to sphincter deficiency and pelvic floor anatomical dysfunction[1]. These defaults may be associated with pelvic-related muscles, nerves, or connective tissue injuries. It is well known that fibroblasts play a core role in tissue repair and extracellular matrix (ECM) production, including strengthening pelvic floor tissue. Studies demonstrated that postpartum recovery after vaginal childbirth induced fibroblast apoptosis and reduced ECM component production, which may lead to SUI conditions [2–5].

Fibroblasts are non-hematopoietic structural cells that can repair adjacent organ structures playing significant roles in fibrosis, cancer, autoimmune disorders, and wound healing[6]. Fibroblasts can produce structural proteins like collagen and elastin for most mesenchymal tissues[7]. The Fibroblasts may also keep tissues healthy by generating and modifying ECMs[6, 8]. Most recently, studies demonstrated that the mesenchymal stem cells (MSCs) secretome significantly stimulates the fibroblast during the healing process of skin wounds[9, 10]. In our previous study, we found that the MSCs secretome can significantly promote the survival and growth of the local periurethral (vaginal side) fibroblast, which can improve urinary incontinence in rat models via injected with MSCs secretome after stimulated birth trauma[11]. The CC motif ligand 7 (CCL7), previously also known as monocyte-chemotactic protein 3 (MCP3), is a
ligand of CCR2 [12, 13]. One of the most important functions is recruiting monocytes to the inflammatory reaction site chemokine’s role in recruiting basophils, T cells, and natural killer cells. The CCL7 is also a chemoattractant for eosinophils [14, 15]. Our previous study demonstrated that CCL7 could promote MSCs engraftment and accelerate urinary incontinence recovery [16]. However, it remains unknown if CCL7 could activate local periurethral fibroblasts by facilitating the secretion of MSCs’ efficient factor. Therefore, in this study, we hypothesised that the CCL7 might recruit needed elements for the local fibroblasts from the MSCs secretomes, which may induce a much faster recovery and stimulate periurethral fibroblasts growth.

Methods And Materials

Human periurethral fibroblasts cell culture and identification

Periurethral fibroblast cells were collected from the local tissue closing the anterior vaginal wall of a 51-year-old patient during an anti-incontinence and prolapse procedure. The tissue was shredded into small pieces 1–3 mm and seeded into a T-25 flask containing 20% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS). Tissue was cultured until cells became visible around the tissue, and when the fusion reached 90% (FIGURE 1A) ask lled with the prepared culturing medium was sent to the company for further immortalisation. Cell immortalisation was done for cell stability and longer-term use. Immortalised cells were cultured with 10% FBS and 1% PS in the DMEM medium. Passaging of cells was performed every 2–3 days in 1:2 dilution. Cells were cultured at 37 degrees C with a 5% CO2 incubator.

Immortalisation of the human periurethral fibroblasts

The primary human fibroblasts were washed three times with PBS, digested with 0.25% trypsin for 3 minutes in an incubator, and then terminated with complete medium (high glucose DMEM + 20% FBS + 1% double antibody). Following centrifugation at 1200rpm for 5 minutes, the cell pellets were resuspended in the medium above and plated in 6cm dishes. At 50% confluence, the cells were infected with lentivirus pGMLV-SV40T-PURO, MOI = 80 (containing 5ug/mL Polybrene to boost the efficacy of lentivirus infection) and cultured overnight in an incubator at 37°C and 5% CO2. The medium was replaced on the second day after the lentivirus infection, and then it was changed every two days for the next four days as the culture continued. The culture was continued for four days after adding 2ug/mL Puromycin to the cell complete medium. The chosen monoclonal cells were digested with 0.25% trypsin and then plated in 6cm dishes. After the cells multiplied and merged, they were routinely passed and grown (NEWGAINBIO Inc. Wuxi, Jiangsu, China) (FIGURE 1B-C).

Immunofluorescence identification

The medium was removed when the human periurethral fibroblasts reached 80% confluence. The cells were washed twice with PBS for 10 minutes each and then treated for 15 minutes at room temperature with 4% paraformaldehyde. The cells were then rinsed twice with PBS for 10 minutes, followed by permeabilisation with 0.1% Triton X-100 at four °C for 15 minutes. Following two 10-minute washes with
PBS, the cells were blocked with 4% BSA for 30 minutes at room temperature. After adding diluted 1:100 Vimentin antibody, the sample was refrigerated overnight at 4°C. The following day, cells were washed three times with PBS for 10 minutes each, anti-FITC secondary antibody 1:100 was added, and cells were incubated at 37°C for 2 hours. In the end, images were viewed and photographed using an inverted fluorescent microscope after being rinsed three times for 10 minutes each with PBS (FIGURE 1D-E)

**Mesenchymal stem cell conditioned medium (MSC-CM) and CC motif ligand 7 conditioned medium (CCL7 + MSC-CM)**

The human MSCs were provided by Shanghai Zhongqiao Xinzhou Biotechnology Co. (ZQ0308, China), maintained in 10% FBS and 1% PS filled t-25 flask with DMEM and cultured in an incubator at 37°C and 5% CO2. Passaging was performed in 2–3 days of 1:2 dilution. MSCs were cultured in 100mm dishes till 80–90% of fusion. For MSC-CM, the medium was replaced with FBS-free culturing medium after washing with PBS three times, while for CCL7 + MSC-CM, the FBS-free medium contained 0.1% of CCL7. Both dishes were incubated at 37 °C with 5% CO2 for 48h, and the conditioned medium was then collected. Both groups conditioned medium was filtered through a 0.22mm sterile filter (Millipore) to obtain a clean, conditioned medium. Centrifugal filter units with a 3-kDa cutoff (Millipore, USA) were used to concentrate the CM by ultrafiltration. Each group prepared three biological replicates mailed to Shanghai Biotree Biomedical Technology Co., Ltd. for further proteomics.

**Proteomics**

Two groups of cell supernatant samples were used in this study, namely the CCL7 + MSC-CM group and the MSC-CM group. Three corresponding biological replicates of each group and a total of six samples were subjected to proteomic analysis. We preprocessed the samples and then used Tandem Mass Tag (TMT) for protein separation and quantification of six samples. The experimental results were then compared with the database, and finally, GO Annotation Enrichment Analysis, Protein-Protein Interaction Network Analysis, Volcano Analysis, Hierarchical Analysis, Clustering Analysis and other bioinformatics analysis were performed (FIGURE 3). According to the proteomics results, we selected the molecules according to the differential protein screening conditions that the unique peptide is ≥ 1, the Foldchange is > 1.2 or < 0.83, and the P-value is < 0.05. We then screened the proteins to study their involvement in the signal transduction pathway related to the enhancement of fibroblast proliferation and migration ability.

**Fibroblasts transfection**

Cell transfection was performed using a GP-transfect-MATE and siRNA according to the manufacturer’s protocol to inhibit EFNA1 ligand-receptor EphA2. In short, when CM added day, fibroblasts were transfected. Following protocol[17] GP-transfect-MATE and siRNA prepared with DMEM were mixed for 15 minutes and then added to the experimental wells. After 4–8 hours of transfection, the medium was replaced with CCL7 + MSC-CM, and further tests were performed.
Cell counting kit-8 (CCK8)

Cell counting kit 8 was used for the cell proliferation test. Cells of 3x100 / well were seeded in 96 well plates overnight, containing 1% FBS culturing medium. Then after thrice washing with PBS, the old medium was replaced with MSC-CM and CCL7 + MSC-CM. For another two groups, siRNA + CCL7 + MSC-CM or CCL7 + MSC-CM was added. Cell proliferation was tested at 24h and 48h by adding 10ul of CCK8 solution in each well and incubating for 1-4h. Absorbance was detected using a microplate reader (Molecular Devices, SpectraMax Plus 384, USA) at 450nm.

Cell migration assay/wound-healing assay

After cells were cultured in the t-25 flask and reached fusion of 100%, cells were digested and seeded in 6 well plates in triplicate for cell migration assay. When the cells formed a confluent monolayer, the medium was replaced with FBS-free DMEM overnight. Next, the scratch was done using a sterile 200 µl pipette tip to create a linear gap in the monolayer. After, it was washed thrice with PBS, and MSC-CM or CCL7 + MSC-CM was added to the respective wells. For another two groups, siRNA + CCL7 + MSC-CM or CCL7 + MSC-CM was added. Four randomly selected points were marked to evaluate the migration area on each well. Images were acquired at 0h, 24h, and 48h using an OLYMPUS CKX53 microscope.

Western blotting

For expression detection after each CM treatment, fibroblasts were seeded in 100mm dishes. Whole proteins (from MSC-CM and CCL7 + MSC-CM treated cells and for another two groups siRNA + CCL7 + MSC-CM and CCL7 + MSC-CM) were extracted and by using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, USA) was determined their concentrations. The proteins on each sample were separated on 10% SDS-PAGE gels and blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, IPVH00010, USA). Quick Block solution was used for blocking according to the manufacturer's requirements. Then membranes were incubated for a night at 4°C with antibodies against GAPDH/B-actin, PI3k, p-PI3k, AKT, p-AKT, and PCNA. The next day after washing with TBST thrice, membranes were incubated with a secondary antibody. The detection of protein bands was tested by chemiluminescence, and the intensity was measured using Image-J.

Reverse transcription-polymerase chain reaction

To choose the best siRNA (Table 1) for transfection of FBs to block the EFNA1 receptor, EphA2, cells were cultured in 12 well dishes in triplicate and then repeated thrice. TRIzol reagent (Ambion, Life Technologies, USA) was used for RNA extraction according to the manufacturer's protocol, then quantified spectrophotometry (DeNovix, DS-11, USA). Using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA), cDNA was obtained. Then cDNA templates were amplified by SYBR Green master mix kit (ABI, USA) and using the primers shown in Table 2. All the samples were repeated thrice, and the program was set as follows: pre-denaturation is conducted at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 15 seconds, and elongation
at 722103 for 45 seconds in a PCR instrument (Thermo Fisher Scientific, ABI QuantStudio 5, USA). The mRNA levels were determined using the $2^{-\Delta\Delta Ct}$ technique and normalised to GAPDH levels.

Table 1
siRNA used for transfection

<table>
<thead>
<tr>
<th>Human EPHA2</th>
<th>sense (5’-3’)</th>
<th>antisense sense (5’-3’)</th>
</tr>
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<tbody>
<tr>
<td>EPHA2-Homo-444</td>
<td>GCUCAAGUUUACUGUACGUTT</td>
<td>ACGUACAGUAAACUUGAGCTT</td>
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<tr>
<td>EPHA2-Homo-733</td>
<td>GUCCGUGUCUACUACAAGATT</td>
<td>UCUUGUAGAGCACGGACTT</td>
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<tr>
<td>EPHA2-Homo-1553</td>
<td>GGAAGUACGAGGUCACUUATT</td>
<td>UAAGUGACCUCGUACUUCCTT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>UGACCUCAAACUACAUUGGUUTT</td>
<td>AACCAUGUAGUUGAGGUCATT</td>
</tr>
</tbody>
</table>

Table 2
Primer sequences used in RT-PCR.

<table>
<thead>
<tr>
<th>GENE</th>
<th>Primer sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>F: GGAGCCAAAAGGGTCATCATCTC R: AGGCATTGCTGATGATCTTGAGG</td>
</tr>
<tr>
<td>EPHA2</td>
<td>F: CCACCACAACATCATCCGCCTA R: CACGCTGAACCTGCGCCCATCTT</td>
</tr>
</tbody>
</table>

Detection and verification of the PI3k and AKT pathway

Alterations of pathways in different CM-cultured fibroblasts were detected by western blotting. The actions outlined above were completed, and antibodies against PI3k (1:1000), p-PI3k (1:1000), AKT (1:1000), and p-AKT (1:1000) were used. For the verification of the effect of PI3k/AKT, CCL7 + MSC-CM-cultured fibroblasts were treated with CCL7 + MSC-CM or CCL7 + MSC-CM + 10 Mµ 1553/733 to test the influence of the PI3k/AKT pathway on proliferation, migration, and gene expression. After following the protocols mentioned above, a CCK-8 assay and a wound-scratch assay were performed.

Statistical analysis

The data were presented as mean ± standard deviation of the mean. All statistical analyses were performed using GraphPad Prism 8.3.0. The two-sided Student’s t-test was used to analyse the differences between the two groups. The one-way analysis of variance was used when more than two groups were analysed. A p-value of less than 0.05 was considered as a significant difference.

Results

Immortalised fibroblasts

According to our previous study, human fibroblasts were used after 5 passages in 6th -8th generations. We decided to immortalize the FBs to further stabilize them and use for longer term. After cell immortalisation, the tests were repeated and yielded satisfying results. Human FBs and immortalised FBs
were identical except for the lifetime. The immortalized FBs live term gave us a more extended use, as long as 14th-18th generations. As these cell lines were not reported before, immortalised cell lines came into work just as well as human tissue collected fibroblasts. (FIGURE 1)

**CCL7 combined with MSC-CM facilitates the proliferation and migration abilities of fibroblasts**

Fibroblasts were isolated from periurethral wall tissue and immortalised. To test whether CCL7 + MSC-CM affected fibroblast migration, the wound-healing assay was performed. Compared to MSC-CM, CCL7 + MSC-CM showed better results in cell migration after 24h and 48h of medium addition (FIGURE 2A). The quantitative analysis of the migratory area is shown in FIGURE 2B. Additionally, a CCK-8 experiment was carried out to investigate the impact that CCL7 + MSC-CM had on cell proliferation. As can be seen in FIGURE 2C, when compared with the group given the MSC-CM, the number of viable fibroblasts, measured by measuring the OD value, increased in the group given CCL7 + MSC-CM after 24 hours and 48 hours respectively.

**Proteomics**

Bioinformatics analysis such as GO Annotation Enrichment Analysis, Protein-Protein Interaction Network Analysis, Volcano analysis, and Hierarchical Clustering Analysis showed significant differences in protein expression levels between the CCL7 + MSC-CM group and the MSC-CM group from multiple perspectives. The results of proteomic visualization facilitated in selecting molecules with significantly different expressed proteins and many associated interacting proteins, and then for the screening proteins, we studied their involvement in the signal transduction pathways related to the enhancement of fibroblast proliferation and migration. (FIGURE 3)

**Ephrin-A1 (EFNA1) protein inhibitor**

EFNA1 is the most commonly seen protein in PI3k/AKT pathway stimulation. To inhibit the EFNA1 protein, siRNA was used to inhibit its most common gene, Ephrin type-A receptor 2 (EphA2). To test between three inhibitors, rt-PCR was performed. EphA2 siRNA of the 733 and 1553 family showed better control of its gene, which was further chosen for protein inhibition (FIGURE 4D).

**CCL7 + MSC-CM regulates collagen metabolism in fibroblasts**

To investigate how CCL7 + MSC-CM influences collagen synthesis and metabolism, the protein levels of collagen expression were analysed by western blotting (FIGURE 2D). The protein expression of MSC-CM and CCL7 + MSC-CM in PI3k/AKT pathway and proliferation cell nuclear antigen were tested. The results of the western blotting assay showed significant changes in the levels of protein expression in the CCL7 + MSC-CM group (FIGURE 2E).
CCL7 + MSC-CM activates fibroblasts via the PI3k/AKT pathway

We studied the involvement of AKT and its upstream PI3k molecules in these events to investigate the alterations in signal transduction pathways associated with enhanced proliferation and migration ability of fibroblasts in response to CCL7 + MSC-CM stimulation. These pathways are associated with enhanced proliferation and migration ability of fibroblasts. Comparing the levels of p-PI3k and p-AKT in fibroblasts cultivated with CCL7 + MSC-CM to those cultured in the MSC-CM, Western blotting demonstrated an increase in the levels of both proteins. In CCL7 + MSC-CM-cultured fibroblasts, treatment with the RNA inhibitor 733/1553 reduced EFNA1 protein expression in PI3k/AKT pathway (FIGURE 4E). Further, it was investigated whether inhibition of EFNA1 affected fibroblasts’ proliferation and migration. As can be seen in FIGURE 4A-B, the application of siRNA to the fibroblasts cultured with CCL7 + MSC-CM inhibited their capacity for cell migration. Similarly, siRNA was able to significantly reduce the capacity of the fibroblasts cultivated in CCL7 + MSC-CM to proliferate (FIGURE 4C). These findings can draw the conclusion that CCL7 + MSC-CM stimulated fibroblasts through the PI3k/AKT pathway.

Discussion

Stress urinary incontinence is thought to be due to neuromuscular damage and a lack of support for pelvic connective tissue, accompanied by fibroblast dysfunction and reduced collagen content [18–20]. With the development of cell therapy, several studies have shown great promise of mesenchymal stem cells (MSC) as a non-surgical treatment for stress urinary incontinence [21–23]. In reality, treatment outcomes vary widely, possibly due to differences in the implantation rate and survival of mesenchymal stem cells implanted into urethral tissue. Migration and transplantation are prerequisites for MSCs to function in injured tissues.

To improve the efficiency of MSCS therapy, previous investigators have found that some chemokines can induce the migration of MSCs and help the recruitment of MSC molecules to the damaged urethra and vaginal tissues and proposed the concept of the combination of chemokines and MSCs therapy [11, 24–29]. The chemokines that induce MSC migration mainly include CCL3 (formerly MIP-1α), CCL4 (formerly MIP-1β), CCL5 (formerly RANTES), CXCL12 (formerly SDF-1) and CCL7 (formerly MCP-3)[30, 31]. This study has conducted a comparative experiment of MSCs with CCL7 as the variable to verify the effectiveness of the combined treatment regimen of chemokine and mesenchymal stem cells, that is, whether chemokine CCL7 increased the implantation and survival of MSCs and promoted the proliferation of periurethral fibroblasts.

The present study is an innovative use of periurethral fibroblasts derived from incontinence patients with anterior pelvic prolapse and immortalised to obtain stable cell line properties. The following experiment is the first of its kind using immortalised fibroblasts in treating stress urinary incontinence with MSCs. Immortalised fibroblasts are as effective in research as primary human fibroblasts. Moreover, compared
with primary human fibroblasts, immortalised fibroblasts have stronger stability and longer service life, showing advantages in experiments.

Through wound healing assay and CCK-8 assay, the data obtained showed that the CCL7 + MSC-CM group had better cell migration results and more vital cell survival ability. Therefore, the experiment further proves that the combined treatment of CCL7 and mesenchymal stem cells conditioned medium were more effective in improving SUI than the MSC-CM group. However, the mechanism by which CCL7 helps MSC implant into damaged tissues is not fully understood. As a result, the molecular regulatory pathways of CCL7 combined with MSCs were closely investigated. Eventually, proteomic analysis was performed on cell supernatant samples from two groups (CCL7 + MSC-CM group and MSC-CM group, respectively, with 3 biological replicates in each group and a total of 6 samples). According to proteomics results, the molecules with significantly differentially expressed proteins were selected and many associated interacting proteins in the two groups and then targeted the proteins to study their involvement in the signal transduction pathways related to the enhancement of fibroblast proliferation and migration ability. After rigorous data comparison, it was noticed that PI3k and AKT were significantly different. Previous studies have shown that PI3k/AKT is one of the most important pathways controlling many cellular processes. One known downstream molecule of PI3k is the AKT kinase, which can affect the cell cycle regulation, transcription, translation, apoptosis and differentiation. Activated AKT functions through the use of an extensive protein network. Later, the experiments were designed for the selected proteins PI3k and AKT and used Western Blot to prove that the CCL7 combined treatment group had higher protein expressions of p-PI3k and p-AKT.

In addition, we investigated EFNA1 (Ephrin-A1), a common protein in the PI3k/AKT pathway. Ephrin type-A receptor 2 (EphA2) signalling regulates multiple cellular processes (proliferation, survival, migration, morphology, intercellular rejection, and adhesion) in embryonic development, angiogenesis, and tumorigenesis. Eph receptors and ephrin ligands control cell-cell interactions in neurological and vascular systems. Ephrin interaction activates Eph kinase, initiating ‘forward’ signalling in receptor-expressing cells. Eph receptors and ephrins are membrane-bound, so they only interact at cell-cell contacts. Without cell-cell interactions, the molecules exist in loosely linked clusters (microdomains), which become more compact upon Eph receptor/ephrin complex formation, producing signalling centres. The structure of an A-class receptor, i.e., EphA2, was found to be the same both by itself and when it is bound to its specific ligand, ephrin-A1. Given the positive association of EphA2 overexpression with aggressive clinical and pathological features in human cancers, researchers have examined the possibility of downregulation of EphA2 in preclinical models. Short interfering RNAs (siRNAs) for gene knockdown are important tools for protein function assessment, gene discovery and drug development and have been applied to silence EphA2 in human cancer cells. Therefore, siRNA was used to inhibit EphA2, the most common gene of ENFA1 (Ephrin-A1). And to test for differences between the three inhibitors, rt-PCR was performed. The results showed that EphA2 siRNA of the 733 and 1553 families had better control over their genes. From the proteomic analysis, it was anticipated that EFNA1 with its receptor EphA2 could be the optimal choice for the following experiment, as determined by its P-value (0.05:0.016935186) and fold-change (> 1.2:1.3365347). Afterwards, EFNA1 protein was further
selected for inhibition. When the expression of ENFA1 in MSC-CM was reduced by inhibitor, the proliferation of treated fibroblasts was reduced, which proved that ENFA1 is a key protein in the proliferation and migration of fibroblasts affected by the combination treatment of CCL7 and MSCs. It was concluded that compared with the MSC-CM group, the combination of cytokine CCL7 and MSC-CM stimulated fibroblast growth more, and CCL7 + MSC-CM stimulated fibroblasts through PI3k/AKT pathway, thereby improving fibroblast dysfunction associated with stress urinary incontinence.

The limitation of the following research is that it only investigated the most likely PI3k/AKT pathway in the mechanism of CCL7 combined with MSCs. But in fact, there may be other signal transduction pathways involved in this treatment, and there may be cross-effects between different signal transduction pathways. And due to the limitations of experimental conditions and factors such as time, only cell experiments were performed. Future research plans to combine animal experiments to conduct further studies to improve the effectiveness of the combined treatment of chemokines and MSCs.

In conclusion, the study displays that the combined treatment of CCL7 and MSCs activates the proliferation and migration ability of periurethral fibroblasts through the PI3k/AKT pathway and modulates the expression levels of the corresponding proteins. Further inhibition of EphA2 733/1553 family receptor of EFNA1 protein PI3k/AKT showed a significantly reduced proliferation and migration of periurethral fibroblasts. This study will help people further understand the combined treatment of mesenchymal stem cells and chemokines and improve CM’s efficiency in treating SUI.

Declarations

Ethical Approval

This study has received ethical approval from the Ethics Committee Boards of The First Affiliated Hospital of Wenzhou Medical University (Ethical review of clinical research 2016 No. 018).

Consent to participate and/or Consent to publish

All authors have agreed on the journal to which the article has been submitted. All authors have agreed to be accountable for all aspects of the work.

Author Contributions

All authors made a significant contribution to the work reported, including conception, study design, execution, acquisition of data, analysis and interpretation, took part in drafting, revising or critically reviewing the article. All authors commented on previous versions of the manuscript, read and approved the final manuscript.

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**Competing of interests**

All authors declared no competing of interest or potential competing of interest

**Availability of data and materials**

The datasets generated during and/or analysed during the current study are available from the Office of Scientific Research at the corresponding author affiliation on reasonable request.

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Figures
Figure 1

Immortalised fibroblasts. (A) Primary periurethral tissue from a patient with urinary incontinence and anterior pelvic prolapse under the light microscope (magnification, ×4). (B) Primary periurethral fibroblasts were observed under a light microscope (magnification ×4). (C) Morphological observation of immortalised fibroblasts (magnification of ×10 & ×20). (D) Primary periurethral fibroblasts stained by immunofluorescence under an inverted fluorescence microscope (magnification of ×10 & ×20). (E)
Immortalised fibroblasts stained by immunofluorescence under an inverted fluorescence microscope (magnification of x10 & x20).

**Figure 2**

CCL7 combined with MSC-CM can promote the proliferation and migration ability of fibroblasts. (A) Wound healing assay of fibroblasts treated with different media at 0, 24 and 48 hours (magnification,
(B) Percentage of migrated areas in different groups. (C) Cell numbers are shown as OD values of fibroblasts treated with different media in the CCK-8 assay. (D) Western Blot showed the expression levels of proteins p-PI3k, PI3k, p-AKT, AKT, PCNA and β-Actin in the two groups of fibroblasts. (E) Shows the relative expression levels (relative to β-Actin) of proteins p-PI3k, PI3k, p-AKT, AKT, and PCNA in two groups of fibroblasts treated with different media. Data are shown as mean ± standard deviation (SD). *P<0.05, **P<0.01, ***P<0.001. MSC-CM, mesenchymal stem cells conditioned medium; CCL7+MSC-CM, mesenchymal stem cell conditioned medium supplemented with CC motif ligand 7.
Figure 3

Proteomics. (A) The classification histogram of GO enrichment analysis shows the number of differentially expressed proteins enriched by GO entries in the CCL7+MSC-CM group and the MSC-CM group. (B) The histone interaction network diagram represents the interaction between different proteins in the CCL7+MSC-CM group and MSC-CM group; red represents up-regulation, blue represents down-regulation; the size of the circle represents the degree of connectivity of differentially expressed proteins, and the degree of connectivity. (C) The volcano plot shows the distribution of differences in protein expression levels between CCL7+MSC-CM and MSC-CM samples. (D) The hierarchical clustering analysis heat map showed a distinct grouping pattern of differentially expressed proteins between the CCL7+MSC-CM group and the MSC-CM group.
Figure 4

siRNA inhibited the enhanced effect of CCL7+MSC-CM on the proliferation and migration of fibroblasts in vitro. (A) Wound scratch assay of fibroblasts treated with different media at 0, 24 and 48 hours (magnification, ×4). (B) Percentage of migrated areas in different groups. (C) Cell numbers are shown as OD values of fibroblasts treated with different media in the CCK-8 assay. (D) RT-PCR shows the mRNA expression of EFNA1 protein after treatment with EphA2 siRNA families 1553, 733 and 444, respectively.
Western Blot showed the expression levels of proteins p-PI3k, PI3k, p-AKT, AKT, PCNA and PCNA in fibroblasts treated with two different media. Shows the relative expression levels (relative to GAPDH) of proteins p-PI3k, PI3k, p-AKT, AKT, and PCNA in two groups of fibroblasts treated with different media. Data are shown as mean ± standard deviation (SD). *P<0.05, **P<0.01, ***P<0.001. CCL7+MSC-CM, mesenchymal stem cells conditioned medium supplemented with CC motif ligand 7; siRNA/siRNA+CCL7-CM, mesenchymal stem cells conditioned medium supplemented with siRNA and CC motif ligand 7.

Supplementary Files

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