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Research Article

# Whole genome mRNA expression profiling revealed multiple deregulated pathways in stromal vascular fraction from erectile dysfunction patients

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**Background:** Stem-cell-based therapies have recently been explored in the field of erectile dysfunction (ED). However, the cellular and molecular phenotype of adipose derived stem cells (ADSCs) stromal vascular fraction (SVF) from ED patients remains largely unknown. Herein we compared the global gene expression profile in the SVF from ED patients and healthy individuals and identified altered signaling pathways between the two groups.

**Methods:** Samples (2–5 g) of abdominal adipose tissue from ED patients ($n = 6$) and healthy individual controls ($n = 3$) undergoing elective cosmetic liposuction were collected. Immediately after removal, SVF was separated using Collagenase type I and type IV protocol. RNA was isolated and microarray experiments were conducted using the Agilent platform. Data were normalized and pathway analyses were performed using GeneSpring software.

**Results:** Our data revealed multiple differentially expressed genes between the ED and control group. Hierarchical clustering based on differentially expressed mRNAs revealed clear separation of the two groups. The distribution of the top enriched pathways for the up-regulated genes indicated enrichment in inflammatory response and T-cell receptor signaling, while pathway analysis performed on the down-regulated genes revealed enrichment in mitogen-activated protein kinase, TGF-β, senescence, FAK, adipogenesis, androgen receptor, and EGF–EGFR signaling pathways in SVF from ED patient.

**Conclusion:** Our data revealed the existence of multiple altered signaling pathways in the SVF from ED patients, which could potentially play a role in the etiology of this disease. Therefore, therapeutic strategies targeting these pathways might provide novel therapeutic opportunity for ED patients.

## Introduction

Erectile dysfunction (ED) is a common clinical disorder that affects primarily men older than 40 years of age [1]. Significant scientific developments throughout the past three decades have extended our indulgent of the physiology and pathophysiology of penile erection. However, in addition to the well-known causes of ED, other factors such as obesity, diabetes, hypertension, and numerous collective lifestyle factors, including lack of physical exercise, lower urinary tract symptoms, have also been linked to the development of ED [1–3].

Presently, the majority of ED patients are treated with phosphodiesterase type-5 inhibitors (PDE5is), such as sildenafil, tadalafil, vardenafil, and avanafil [4]. In addition, several other treatment options exist

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**Table 1 Clinical characteristics for ED patients and healthy controls**

| Patient no. | Age | Co-morbid conditions | Body weight (kg) |
|---|---|---|---|
| | | **ED patients** | |
| 1 | 29 | ED, DM | 68 |
| 2 | 47 | ED, no other comorbidity | 81.5 |
| 3 | 34 | Left testicular cancer, postradical orchiectomy, ED | 113.2 |
| 4 | 68 | Prostate cancer, postradical prostatectomy, ED | 76 |
| 5 | 53 | DM, HTN, smoker, right renal cancer, postradical nephrectomy, ED | 90.3 |
| 6 | 42 | Hypothyroidism, primary infertility, ED | 95 |
| | | **Healthy controls** | |
| 1 | 34 | Smoker, bilateral varicoceles, no other medical problems, no ED | 126.1 |
| 2 | 35 | No other medical problems, no ED | 86 |
| 3 | 30 | No other medical problems, no ED | 75 |

Abbreviations: DM, diabetes mellitus; ED, erectile dysfunctions; HTN, hypertension.

for ED, including lifestyle modifications and surgical intervention. With the exclusions of lifestyle modification and revascularization procedures, these methods just treat the indications of ED, proposing symptomatic assistance rather than a remedy for the underlying disease progression [5,6].

Therefore, a critical assessment of the contemporary state of knowledge is crucial to deliver perspective for future research and development of novel therapies. Recently, stem-cell-based therapies have gained attention as a potential substitute in the prevention of ED in various animal models. Interestingly, embryonic stem cells transduced with brain-derived neurotrophic factor (BDNF), adult bone marrow and adipose-derived stem cells were capable of stabilizing erectile function through intracavernous injection [7–10].

Preliminary clinical trials on postradical prostatectomy erectile dysfunction (pRP-ED) patients with bone marrow and adipose-derived stem cells have been reported with encouraging results [11,12].

However, the cellular and molecular phenotype of stem cells between control and ED patients remains largely unknown. Therefore, herein we compared the global messenger RNA (mRNA) expression profile in the SVF from ED patients and healthy individuals to identify the altered signaling pathways between the two groups.

# Methods

## Subjects

The study was approved by the Ethical Committee of King Khalid University Hospital and all the ED patients and healthy individual (control) involved gave informed consent. Samples (2–5 g) of abdominal adipose tissue obtained from abdominal subcutaneous adipose tissue (SAT) of six ED patients, aged from 29 to 68 and healthy individuals control, aged from 30 to 35 undergoing elective open-abdominal surgery (cosmetic liposuction) were collected (Table 1). None of the control individuals presented any chronic disease, diabetes, metabolic syndrome, or altered biochemical parameters that could indicate adipose tissue alterations. Immediately after removal, biopsies were washed in PBS and processed for the separation of SVF.

## Isolation of SVF

Stromal vascular fraction (SVF) isolation was carried out in accordance with our previously published protocols [13]. In brief, freshly isolated SAT biopsies and liposuction were collected in PBS and washed twice to eliminate peripheral blood. Next, samples were incubated in DMEM medium with 41% Collagenase type I and type IV (Gibco-Invitrogen, U.S.A.) for 45 min with gentle agitation at 37°C. After inactivation of collagenase by culture medium DMEM, undigested tissue was removed by filtering through a sterile 100 μm pore Cell Strainer (BD Falcon, CA, U.S.A.) and centrifuged at 600×***g*** for 10 min to separate the floating mature adipocyte layer from the pelleted SVF. SVF was resuspended in DMEM, filtered through a 40 μm pore Cell Strainer and centrifuged at 400×***g*** for 5 min. Pelleted SVF was resuspended in 500 μl of erythrocyte lysis buffer (RBC Lysis Solution, Puregene, MN, U.S.A.) and incubated for 3 min at room temperature. After centrifugation at 400×***g*** for 10 min, SVF was frozen in liquid nitrogen and stored at −80°C until RNA extraction.

**Table 2 List of SYBR green primers used in current study**

| No. | Name | Sequence |
|---|---|---|
| 1 | GAPDH | 5′ CTGGTAAAGTGGATATTGTTGCCAT |
| | | 5′ TGGAATCATATTGGAACATGTAAACC |
| 2 | VEGFA | TCACCAAGGCCAGCACATAG |
| | | CGGCTTGTCACATTTTTCTTGTC |
| 3 | JUN | TGAGTGACCGCGACTTTTCA |
| | | TTTCTCTAAGAGCGCACGCA |
| 4 | IGFI | 5′ TCAGCAGTCTTCCAACCCAA |
| | | 5′ TGGTGTGCATCTTCACCTTCA |
| 5 | PDGFRA | 5′ GACTAGTGCTTGGTCGGGTC |
| | | 5′ CAGGTTGGGACCGGCTTAAT |
| 6 | FOSB | 5′ GCGCCGGGAACGAAATAAAC |
| | | 5′ CAACTGATCTGTCTCCGCCT |
| 7 | LIF | 5′ GCCACCCATGTCACAACAAC |
| | | 5′ CCCCCTGGGCTGTGTAATAG |
| 8 | ZAP70 | CCTGTACGTCCCCAGGTTTC |
| | | CCGTAGAAGAAGGGCAGGTG |

## Gene expression microarray

RNA isolation and microarray analyses were carried out in accordance with our previously published protocols [14]. In brief, RNA was isolated using Total Tissue RNA Purification Kit from Norgen-Biotek Corp. (Thorold, ON, Canada) and were quantified using NanoDrop 2000 (Thermo Scientific, Wilmington, DE, U.S.A.). Total RNA was labeled and then hybridized to the Agilent Human SurePrint G3 Human GE 8 × 60k mRNA microarray chip (Agilent Technologies). All microarray experiments were conducted at the Microarray Core Facility (Stem Cell Unit, Department of Anatomy, King Saud University College of Medicine). Data were subsequently normalized and analyzed using GeneSpring 13.0 software (Agilent Technologies). Pathway analyses were conducted using the Single Experiment Pathway analysis feature in Gene Spring 13.0 (Agilent Technologies). Twofold cut-off with $P < 0.02$ was used. Target prediction was conducted using a built-in feature in Gene Spring 13.0 based on Target Scan database.

## Gene validation by qRT-PCR

Gene expression levels were validated in control and ED patients SVF cells. The procedure was performed in accordance with our previously published protocols [15]. In brief, SYBR Green-based qRT-PCR was performed using Applied Biosystems ViiA Detection system. 500 ng of total RNA was reverse transcribed using High Capacity cDNA Reverse Transcript Kit (Part No: 4368814; ABI) according to the manufacturer's protocol. Relative levels of mRNA were determined from cDNA using real-time PCR (Applied Biosystems ViiA 7 Systems). Primer sequences used in the current study were VEGFA, PDGFRA, FOSB, JUN, IGF1, and LIF are listed in Table 2. The relative expression level was calculated using $-\Delta\Delta$CT. GAPDH was used as an endogenous control.

# Results

The present study composed of six ED patients, aged 29–68 and three healthy individuals; aged 30–35 undergoing elective open-abdominal surgery (cosmetic liposuction) were collected. Routine clinical evaluations were done for both groups including history, physical examination, basic lab testing, and IIEF questionnaire. The clinical characteristics of ED patients and healthy individuals included in current study are presented in Table 1. There was no significant difference in glucose, cholesterol, HDL, LDL, triglycerides, hemoglobin, and white blood count between the ED and control group (Supplementary Figure S1). ED patients had slightly lower LDL and platelets count compared with health controls (Supplementary Figure S1).

## Multiple dysregulated pathways in SVF from ED patients

To understand the molecular alterations in SVF from ED patients, we executed global mRNA expression profile comparing SVF from ED patients with SVF obtained from healthy individuals (control). As shown in Figure 1, hierarchical clustering based on differentially expressed mRNAs revealed clear separation of the two groups, where multiple

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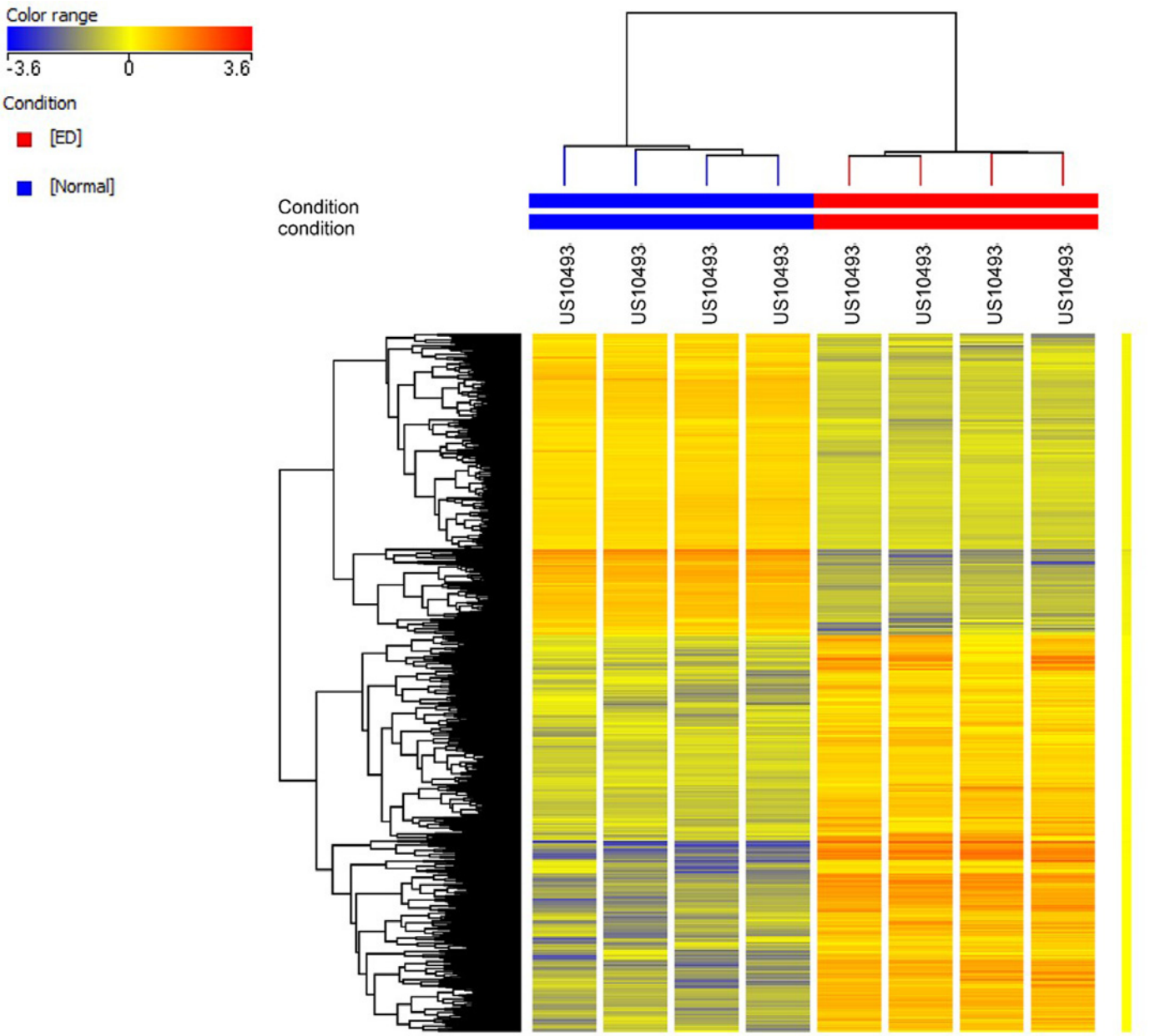


**Figure 1. Differentially expressed genes in ED**

Hierarchical clustering of pooled ED and pooled healthy individual control samples based on differentially expressed mRNA levels. Each column represents a replica and each row represents a transcript. Expression level of each gene in a single sample is depicted according to the color scale.

up-regulated and down-regulated transcripts in the ED patients were observed. The distribution of the top 15 enriched pathways for the up-regulated genes and top 20 enriched pathways for the down-regulated genes in SVF from ED patient are shown in Figure 2. Pathway analysis on the down-regulated genes revealed significant enrichment in several signaling pathways including mitogen-activated protein kinase (MAPK), TGF-β, focal adhesion, adipogenesis, androgen receptor, EGF–EGFR, regulation of actin cytoskeleton, circadian clock, IL-4, neural crest, Wnt, and RANKL-RANK, while inflammation and immune repose were the predominant pathways altered in the up-regulated gene in SVF from ED patients. Up (Inflammatory response: ZAP70) and down (Angiogenesis: VEGFA, PDGFRA;

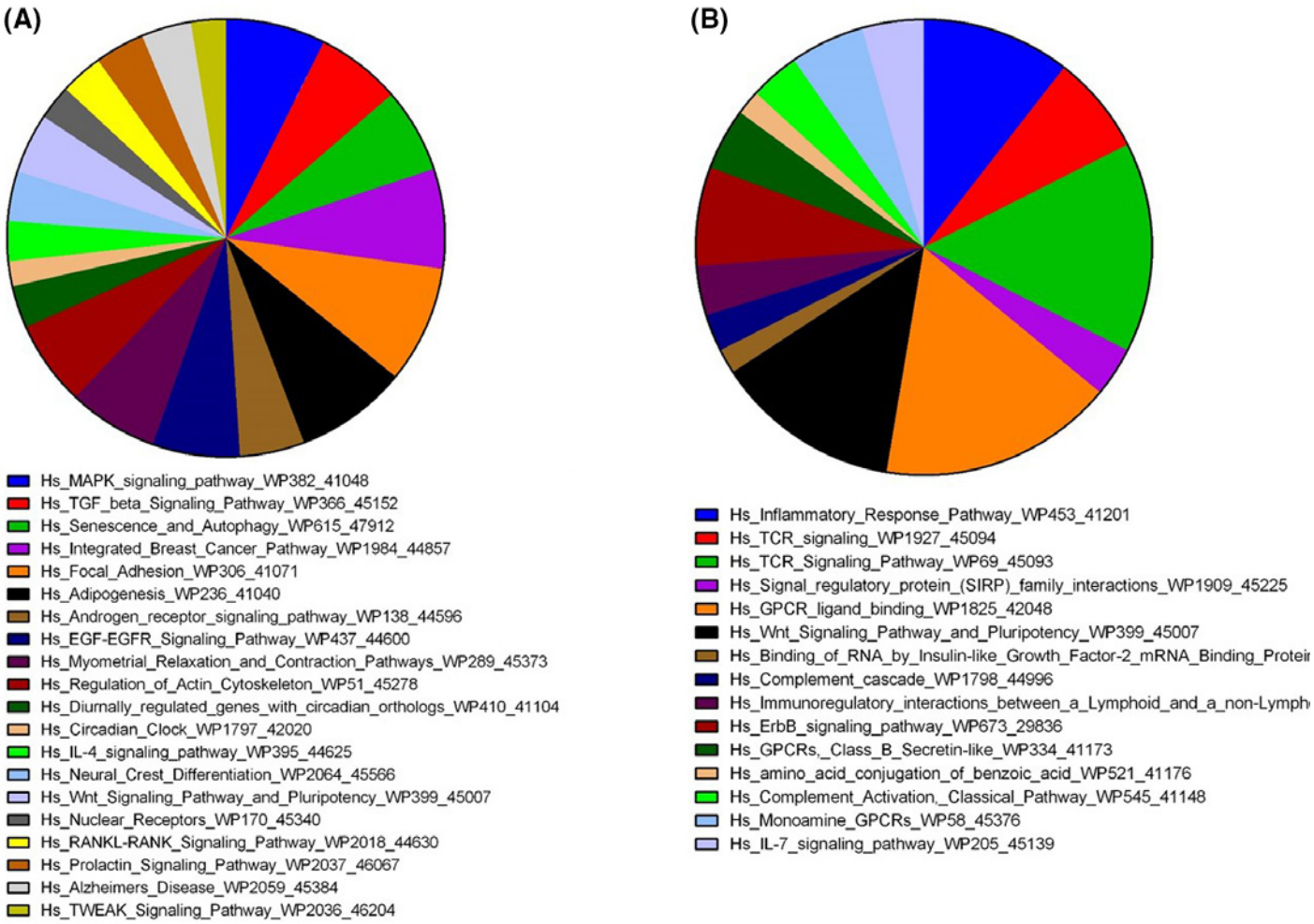


**Figure 2. Pathway analysis of differentially expressed genes in ED patients**

(**A**) Pie chart illustrating the distribution of the top 20 pathway designations for the down-regulated genes in ED patients. (**B**) Pie chart illustrating the distribution of the top 15 pathway designations for the up-regulated genes in ED patients. The pie size corresponds to the number of matched entities.

FAK pathway: PDGFRA, JUN; TGF-β signaling: FOSB, JUN; Adipogenesis: IGF1, LIF) regulated genes from the microarray data were subsequently validated using quantitative reverse transcription-PCR (qRT-PCR) (Figure 3). The FAK and angiogenesis signaling pathways are illustrated in Supplementary Figures S2 and S3.

Furthermore, our analysis on up-regulated genes revealed significant enrichment in numerous signaling pathways including inflammatory response, T-cell receptor (TCR), immunoregulatory, complement activation and IL-7. The inflammatory response signaling pathway is illustrated in Supplementary Figure S4.

# Discussion

Cumulative evidence in the literature suggested multi-lineage differentiation potential for adult stromal stem cells derived from different tissues [16,17], making them an attractive candidate for regenerative medicine. A number of stem-cell-based therapies for ED has recently investigated the therapeutic potential of transplanted adipose derived stem cells (ADSCs) or bone marrow stem cells (BMSCs) via intra-cavernous injection in animal models [7–10]. In humans, 15 studies were found so far in the clinicaltrails.gov database, pertaining to adult stem cell treatment for ED patients (Supplementary Table S1). Among those, four studies utilizing autologous adult stem cells were completed; however, no outcome data are available thus far. Six studies are ongoing and the status of two studies showed withdrawn, two studies are active but not recruiting and finally the status of two studies is unknown (Figure 4).

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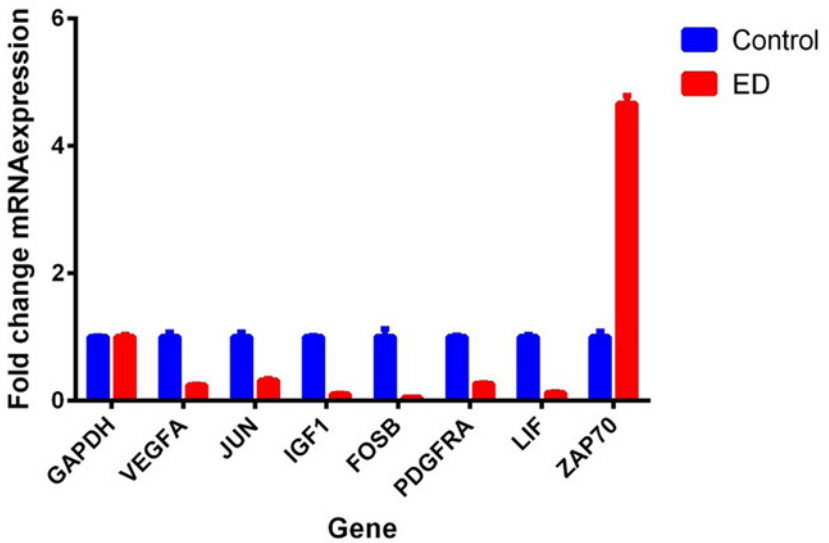


**Figure 3. Validation of differentially expressed genes in ED using qRT-PCR**

Expression levels of selected genes from the microarray data with significant enrichment in the following pathways was validated using qRT-PCR, $n = 3$. **Angiogenesis:** VEGFA, PDGFRA; **FAK pathway**: PDGFRA, JUN; **TGF-β signaling:** FOSB, JUN; **Adipogenesis:** IGF1, LIF; **Inflammatory response:** ZAP70.

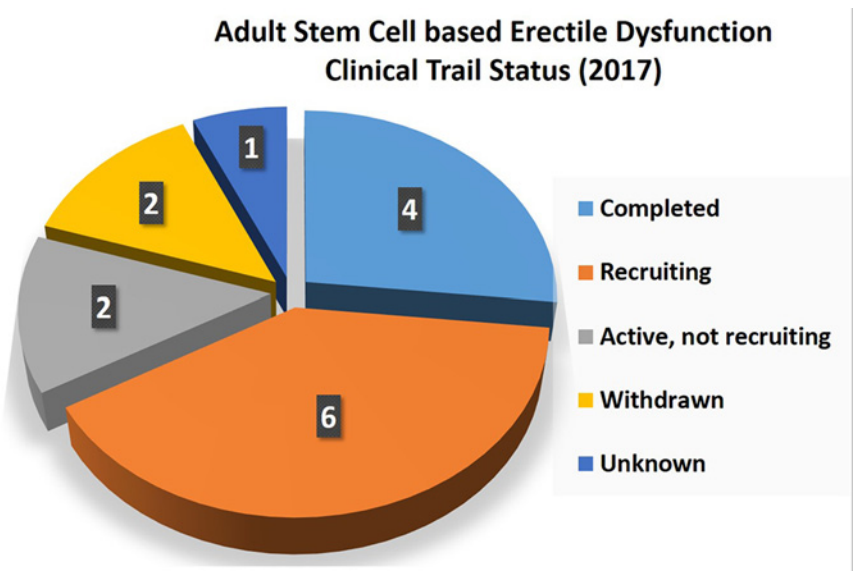


**Figure 4. D pie chart elucidating the adult stem-cell-based clinical trial status for erectile dysfunction (2017)**

Predominantly ADSCs were utilized for the most of these clinical trials, followed by bone marrow, umbilical cord, Wharton jelly, and placenta derived stem cells (Supplementary Table S1).

SVF represents a mixed cellular components made of a combination of ADSCs, endothelial precursor cells, endothelial cells, fibroblasts, macrophages, interstitial cells, smooth muscle cells, lymphocytes, pericytes, and pre-adipocytes among others [18–20]. Both cultured ADSCs and uncultured SVF were shown to exert similar effects in recovering penile erection in rat model of cavernosal nerve injury; however, SVF was superior to ADSCs in terms of histomorphometric changes like endothelial nitric-oxide synthase, smooth muscle/collagen ratio and von Willebrand factor expression [21]. Given the lack of concrete data on the effect of adipose tissue-derived SVF in treating ED in humans, we sought to determine the gene expression signature and signaling pathways of SVF fraction obtained from ED patients compared with healthy individuals to provide more insight into the suitability of SVF from ED patients for autologous transplantation and possibility to enhance their therapeutic efficacy.

Our data highlighted remarkable difference in the gene expression profile of SVF obtained from ED compared the SVF from healthy individuals. We observed significant enrichment in inflammatory and immune response in the up-regulated genes from ED patients, suggesting possible involvement of the immune system in this disease. Our data are in agreement with earlier studies correlating increased levels of inflammatory mediators (such as IL-6) in the circulation and the presence and severity of ED [22]. On the other hand, pathway analysis performed on the down-regulated genes in ED patient's SVF revealed enrichment in MAPK, TGF-β, senescence, FAK, adipogenesis, androgen receptor, and EGF–EGFR signaling pathways. Our previous studies highlighted crucial role for several of the identified pathways in regulating MSC differentiation [23,24]. For instance, integrin-associated FAK regulates cell survival, adhesion, and stem cell maintenance and protects stem cells from apoptosis, detachment, and differentiation [25]. The MAPK pathway incorporates various signalling cascades, of which the Ras-Raf-Mek-ERK1/2 is one of the most dysregulated in cancer and plays important roles during normal physiology including cell proliferation, differentiation, survival, transformation, development, apoptosis inflammatory and other stress responses [26,27]. Down-regulation of MAPK and FAK pathways in ED patients indicates the poor physiological functions including angiogenesis and migration with inflammation and stress response.

Insufficiency of androgen disrupts cellular-signaling pathways and leading to abnormal erectile physiology [28], corroborating our findings on the down-regulation of androgen receptor pathway in SVF from ED patients. Moreover, well identified circadian clock pathway also been down-regulated in ED patients, this clock is synchronized by several environmental stimuli, mainly the light–dark (LD) cycle. Presently, sildenafil, vardenafil and tadalafil have been prescribed for ED to inhibit the phosphodiesterase (PDE5) and increase the low-intensity light-induced circadian responses [29]. In our data, we observed several of the ED patients to be obese or to have other metabolic disorders. Previous study demonstrated significant differences between subcutaneous and visceral/omental fractions of adipose tissue, which were associated with parameters such as BMI, BAI, and/or WHR values [30,31] Therefore, it is plausible that the differences obtained in current study are in part due to other pathological conditions associated with ED such as increase DM and obesity.

In conclusion, our data suggest the existence of multiple altered signaling pathways in the SVF from ED patients compare to healthy individuals, which could potentially play a role in the etiology of this disease with potential implications on autologous SVF transplant for ED patients. Weather these changes are drivers or consequences of ED remains to be elucidated. Therefore, therapeutic strategies targeting these pathways might provide novel therapeutic opportunity for ED patients.

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## Competing interests

The authors declare that there are no competing interests associated with the manuscript.

## Author contribution

R.V.: collection and/or assembly of data, manuscript writing; M.M.: collection and/or assembly of data; A.A.: concept and design; A.A.: collection and/or assembly of data, M.H.: collection and/or assembly of data; D.A.: collection and/or assembly of data; R.A.: collection and/or assembly of data; S.B.: collection and/or assembly of data, concept and design, manuscript writing; N.M.A.: data analysis, concept and design, manuscript writing; M.A. concept and design, funding. R.A.: collection and/or assembly of data.

## Abbreviations

ADSC, adipose derived stem cell; ED, erectile dysfunction; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; HDL, high density lipoprotein; LDL, low density lipoprotein; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; qRT-PCR, quantitative reverse transcription-PCR; SAT, subcutaneous adipose tissue; TGF-β, transforming growth factor beta.

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