In vitro and *in vivo* lineage conversion of bone marrow stem cells into germ cells in experimental Azoospermia in rat

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Abstract

The present study is conducted to assess *in vitro* transdifferentiation of bone marrow derived mesenchymal stem cells (MSCs) into germ cells and *in vivo* transdifferentiation into spermatids and spermatocytes in the seminiferous tubules of azoospermic rats. Forty eight male rats were included in the study and were divided into: group 1; control rats, group 2; experimental azoospermic rats, group 3; azoospermic rats received undifferentiated MSCs. Group 4; azoospermic rats received transdifferentiated MSCs into germ cells. Quantitative real time PCR was conducted to assess gene expression of a primordial germ cell marker (VASA), stem cell specific markers (Oct4, SSEA-1 and SSEA-3), specific molecular markers of germ cells (c-Kit, Daz1); premeiotic marker (Daz1) and post-meiotic markers (c-Kit, Stra 8). Histopathological examination of rat testicular tissue was also conducted. Results revealed that 12 weeks after transplantation, fluorescent labeled MSCs were detected in the seminiferous tubules of the testes of group 3 and group 4 rats. In group 3 and 4, stem cell specific markers; Oct4, SSEA-1 and SSEA-3 were detected. In group 4, genes of primordial germ cell marker; VASA, premeiotic marker; Daz1and post-meiotic markers; c-Kit, Stra 8 were expressed. Daz1 was also expressed in group 3 rats to a significantly lower extent in comparison to group 4 rats. Spermatocytes and spermatids were detected in testicular tissue of group 4 rats. In conclusion, MSCs have potentials for *in vitro* transdifferentiation into germ cells and *in vivo* transdifferentiation into spermatids and spermatocytes.

Introduction

Spermatogenesis is generated by male germinal stem cells as evidenced by regeneration of spermatogic cells after transplantation of total testicular germ cells in the depleted testis.[1,2] Several studies have assessed the potential of reproducing germ cell differentiation, or gametogenesis, *in vitro*.[3] Under appropriate conditions, bone marrow stem cells (BMCs), embryonic stem cells (ESCs) could be induced to transdifferentiate toward the germ cell lineage.[4-6]

Several breakthroughs discovery concerning the *in vitro* derivation of male and female germ cells from stem cells have challenged the field of reproductive biology.[7-10] Mouse bone marrow mesenchymal stem cells (MSCs), grown *in vitro* in the presence of retinoic acid, were found to express germ cell markers.[11,12] A similar transdifferentiation process was described for human bone marrow cells.[13,14]

Nayernia *et al.*[15] stated that BMS cell-derived germ cells expressed the known molecular markers of primordial germ cells; fragilis, stella, Rnf17, Mvh and Oct4; and molecular markers of spermatogonial stem cells and spermatogonia; Rbm, c-Kit, Tex18, Stra8, Piwil2, Dazl, Hsp90alpha, beta1- and alpha6-integrins. Another study has demonstrated that adult bone marrow stem cells can differentiate into Leydig cells in rat testes.[9] Lue *et al.*[12] provided evidence showing that adult bone marrow cells injected into seminiferous tubules or interstitial spaces of busulfan treated mice are able to differentiate into germ cells as evidenced by the detection of VASA marker and they are also able to differentiate into Sertoli and Leydig cells as evidenced by the detection of follicular stimulating hormone receptors and cytochrome P450 (P450scc) side chain cleavage enzyme within the seminiferous tubules.

The present study was conducted to assess transdifferentiation potentials of MSCs into germ cells *in vitro* and transdifferentiation potentials of MSCs into spermatids and spermatocytes in the seminiferous tubules of azoospermic rats.

Materials and Methods

Animal studies

The *in vivo* study was conducted on forty eight Cur1:HEL1 male albino rats, of an average weight 180-200 gm, age 60 days. Rats were inbred strain in the experimental animal unit, Faculty of Medicine, Cairo University. Animals were maintained in an air-conditioned animal house with specific pathogen-free conditions, and were subjected to a 12:12-h daylight/darkness and allowed unlimited access to chow and water. They were maintained according to the standard guidelines of Institutional Animal Care and Use Committee and after Institutional Review Board approval.

Rat were equally divided into the following groups: group1 involved control rats received phosphate buffer saline, group 2 included rats with experimental azoospermia induced by administration of a single intraperitoneal dose of busulfan (GlaxoSmithKline Inc., Mississauga, Ontario, Canada) (35 mg/kg), group 3 included azoospermic rats that underwent transplantation of 1×10^8 undifferentiated MSCs into the testis 2 weeks after induction of azoospermia. Group 4 involved azoospermic rats that underwent transplantation of 1×10^8 differentiated MSCs into germ cells 2 weeks after induction of azoospermia. The injection procedure was conducted as previously decribed.[16] All recipient rats were kept under anesthesia by intraperitoneal administration of Xylazine (20 mg/kg), Ketamine (5 mg/kg), and Athropine (0.04 mg/kg). Microinjection needles 100 µL were used for MSCs transplantation. The testes were surgically exteriorized through a midline abdominal incision, and MSCs suspensions (1×10^8) were injected using microinjection needles. Small incision was made 5 mm from the efferent bundles' junction with the testis. The tip of the microinjection needle was inserted into the bundles and then gently pushed toward the rete testis to enter the seminiferous tubules as described by Ogawa *et al.*[16]

Twelve weeks after MSCs transplantation, animals were euthanized and testicular tissues were collected from all animals and the following parameters were assessed: Testicular histopathological examination, detection of the labeled bone marrow derived stem cells by using PKH26 Red Fluorescent Cell Linker Kit (Sigma Aldrich, St. Louis, MO,

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USA), detection of gene expression of a primordial germ cell marker (VASA), stem cell specific markers (Oct4, SSEA-1 and SSEA-3), a premeiotic marker (Daz1) and post-meiotic markers (c-kit, Stra 8) by real time quantitative PCR.

Animal carcasses were disposed of by incineration in accordance with guidelines stated by Institutional Animal Care and Use Committee (IACUC) guidebook found at NIH website http://www.grants.nih.gov/grants/olaw/guidebook.pdf

In vitro study

Preparation of bone marrow-derived mesenchymal stem cells

BM-derived MSCs were collected from forty eight Cur1:HEL1 male albino rats, of an average weight 180-200 gm, age 60 days. Femurs and tibias were flushed with Dulbecco's phosphate-buffered saline (PBS) (Gibco, catalogue number 14190-240). After filtering the cells through a 70-μm nylon mesh filter, nucleated cells were isolated with a density gradient (Ficoll/Paque, Pharmacia, Piscataway, NJ, USA). The selected cell population was dissociated via trypsin-EDTA treatment, and ~1×10^6 cells were placed in 60 mm dishes with DMEM/F12 (Millipore Corp., Billerica, MA, USA) supplemented with 10% FBS (Millipore), 0.5 mM β-mercaptoethanol (Sigma Aldrich), 20 ng/mL basic fibroblast growth factor (bFGF; Chemicon, Millipore Corp., Billerica, MA, USA), 1000 unit/mL human leukemia inhibitory factor (LIF; Invitrogen, Carlsbad, CA, USA). The seeded cells were cultured in an incubator at 36.5°C and 5% CO_2 at a relative humidity of 60%.[14] For *in vitro* transdifferentiation of MSCs retinoic acid (RA, Sigma Aldrich) treatment was conducted, the collected BMCs were placed in 60 mm non-coated dishes with DMEM/F12 supplemented with 10% knockout serum supplement, 0.5 mM β-mercaptoethanol, 20 ng/mL of bFGF and 1000 unit/mL of LIF for 1 day, after which the knockout serum was replaced with FBS. Cultures were maintained in the above medium for an additional 2 weeks, and the medium was exchanged every three days with the above medium containing 10^{-6} M of RA (Sigma Aldrich) for 10 days.[17]

Characterization of bone marrow-derived mesenchymal stem cells

Cells were characterized as being MSCs by their morphology, adherence, and their power to transdifferentiate into osteocytes and chondrocytes.[18,19] Differentiation into osteocytes was achieved by adding 1-1000 nM dexamethasone (Invitrogen), 0.25 mM ascorbic acid (Invitrogen), and 1-10 mM beta-glycerophosphate (Sigma Aldrich) to the medium. Differentiation of MSCs into osteoblasts was achieved through morphological changes, Alzarin red staining of differentiated osteoblasts and RT-PCR gene expression of osteonectin in differentiated cells. Differentiation into chondrocyte was achieved by adding 500 ng/mL bone morphogenetic protein-2 (BMP-2; R&D Systems, Minneapolis, MN, USA) and 10 ng/mL transforming growth factor b3 (TGFb3) (Peprotech, London, UK). Each specific differentiation medium was changed every 2–3 days for 3 weeks.[19] *In vitro* differentiation into chondrocytes was confirmed by morphological changes, Alcian blue staining of differentiated chondrocytes and RT-PCR of Collagen II gene expression in cell homogenate. Total RNA was isolated from the differentiated MSCs using Trizol reagent (Invitrogen). RNA concentrations were measured by absorbance at 260 nm with a spectrophotometer, and 2 μg total RNA was used for reverse transcription using Superscript II reverse transcriptase (Invitrogen). The cDNA was amplified using Taq Platinum (Invitrogen). Osteonectin gene and collagen (II) primers used were designed according to the following oligonucleotide sequence: sense, 5'GTCTTCTAG-CTTCTGGCTCAGC-3'; antisense,5'-GGAGAGCTGCTTCTCC CC-3' (uniGene Rn.133363) and sense, 5'-CCGTGCTTCTCA-GAACATCA-3'; antisense, 5'-CTTGCCCCATT CATTTGTCT-3' (UniGene Rn.107239). The RNA templates were amplified at 33 to 45 cycles of 94°C (30 s), 58°C to 61°C (30 s), 72°C (1 min), followed with 72°C for 10 min. PCR products were visualised with ethidium bromide on a 3% agarose gel. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected as housekeeping gene to examine the extracted RNA integrity. CD29 gene expression was also detected (Unpublished Data) by RT-PCR as a marker of MSCs.[14]

Labeling of bone marrow-derived mesenchymal stem cells

Stem cells were labeled with PKH26; PKH26 is a red fluorochrome. It has excitation (551 nm) and emission (567 nm) characteristics compatible with rhodamine or phycoerythrin detection systems. In the current work, MSCs cells were labeled with PKH26 according to the manufacturer's recommendations (Sigma). In brief, the procedure involves incubation of 2 mL staining solution containing final concentrations of 2×10^{-6} M of PKH26 and 2×10^7 cells/mL. The cells/dye suspensions were incubated for 1-5 min with periodic mixing. Staining is almost instantaneous and was stopped by incubation for 1 minute with an equal volume (2 mL) of 1% BSA to allow binding of excess dye. Cells were centrifuged at 400 × g for 10 min at 20-25 °C to remove the supernatant. Cell pellets were washed twice with complete medium to ensure removal of the dye then resuspended in complete medium.

Cells were injected into the testicular tissue. After 12 weeks, testicular tissue was examined with a fluorescence microscope to detect the cells stained with PKH26.

RNA isolation and qualitative RT–PCR

Testicular homogenate was prepared by homogenizing 100 mg testicular tissue with 500 μL RNA lysis buffer (Promega Corp., Madison, WI, USA). Total RNA was extracted from testicular tissue homogenate using RNeasy purification reagent (Promega). cDNA was generated from 5 μg of total RNA extracted with 1 μL (20 pmol) antisense primer and 0.8 μL superscript AMV reverse transcriptase (Promega) for 60 min at 37°C.

Quantitative Real-time PCR

Quantitation of gene expression was conducted using universal probe library sets based real time PCR (Roche Diagnostics, Basel, Switzerland). Selection of genes specific probes and primers were done using the online ProbeFinder software and the real time PCR design assay of Roche Diagnostics found at their website (Table 1).[20] Each sample was analyzed at duplicate.

Hypoxanthine phosphoribosyl transferase 1 (Hprt1) was used as a positive control housekeeping gene. FastStart Universal Probe Master mix was used in LightCycler® 480 Instrument (Roche Applied Science) according to the manufacturer's recommendations. Briefly, in the LightCycler® 480, a total reaction volume of 20 μL was prepared, of which 2 μL of starting RNA material was included for RT-PCR, a final concentration of 0.5 μM of each forward and reverse primer and 0.2 μM of the TaqMan probe was used. Cycling conditions involve reverse transcription at 50°C for 30 min; enzyme activation at 95°C for 15 min, followed by 50 cycles of 95°C for 10 s and 60°C for 60 s. LightCycler® 480 RT-PCR data were analyzed using Light Cycler1.2 version 3.5 software using the second derivative maximum method. Successfully amplified targets are expressed in Ct values, or the cycle at which the target amplicon is initially detected above background fluorescence levels as determined by the instrument software. Each sample RT-PCR was performed minimally in duplicate, and the mean Ct value with standard deviation reported.

Statistical Analysis

Statistical Package for Social Studies (SPSS) program version 16.0.1 (SPSS Inc., Chicago, IL, USA) was used. Numerical data were expressed as mean ± standard deviation. For comparisons between treatment groups, the null hypothesis was tested by a single-factor ANOVA. Multiple comparisons were con-

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ducted using Tukey-Kramer test. Comparisons were considered statistically significant if $P<0.05$.

Results

In the present study we characterized MSCs by their morphology (Figure 1).

The pluripotency of bone marrow derived mesenchymal stem cells (MSCs) was evidenced by the transdifferentiation ability of MSCs into osteocytes and chondrocytes (Figure 2).

Stem cell specific markers (Oct4, SSEA-1 and SSEA-3) were expressed in group 3 and 4 rats (Figure 3).

In vitro transdifferentiation of MSCs into germ cells was induced by retinoic acid (RA) and was evidenced by the significant upregulation of gene expression of the primordial germ cell marker (VASA), specific molecular markers of germ cells (c-kit, Daz1) (Figure 4).

Fluorescent-labeled transdifferentiated MSCs reside in the seminiferous tubules of groups 3 and 4 rats 12 weeks after transplantation as demonstrated by detection of labeled MSCs in the testicular tissues of group 3 and group 4 rats (Figure 5).

In vivo transdifferentiation of MSCs into premeiotic and postmeitic spermatogenic cells was evidenced by the significant upregulation of gene expression of a premeiotic marker (Daz1) and post-meiotic markers (C-kit, Stra 8) (Figure 6) in the testicular tissues of group 4 rats.

Histopathological examination of testicular tissue revealed appearance of spermatocytes and spermatids in the testicular tissue of group 4 rats that received differentiated MSCs. Spermatocytes and spermatids were not detected in groups 2 and 3 rats (FigureS 7 and 8).

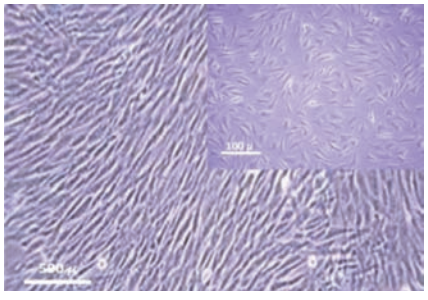

Figure 1. Undifferentiated mesenchymal stem cells in culture for 2 weeks with 2 different magnification scales.

Discussion

The present study was conducted to assess transdifferentiation potentials of bone marrow derived MSCs into germ cells *in vitro* and into pre-meiotic and post meiotic spermatogenic cells *in vivo* in experimental azoospemia in rats. MSCs were isolated by the standard protocol and their pluripotency were confirmed by their transdifferentiation into osteoblasts and chondroblasts *in vitro*. Among these transdifferentiation capabilities of MSCs, the generation of germ cells is one of their most interesting characteristics. Although the issue of spermatogenesis from MSCs is somewhat controversial, several previous reports have discovered evidence that BMC can transdifferentiate into male germ cells.[12,13,17-21] Nayernia *et al.*[9] demonstrated for the first time that ESC-derived germ cells were able to generate offspring mice, and also showed that murine BMC could differentiate into male germ cells. Recently, Lue.[12] reported that BMC transplanted into the testes of a busulfan-treated infertility mouse model appeared to differentiate into

Table 1. Primer sequences used for real time PCR.

Oct-4	
(POU class 5 homeobox 1 , Pou5f1)	5' CCTGCAGCAGATCACTAGCAT 3'
GenBank® Accession Number NM_001009178.1	5' CACTCGAACCACATCCCTCT 3'
Stimulated by retinoic acid gene 8 (Stra8)	5' CAGATCATCGAGTTTTTCAAAGG 3'
GenBank® Accession Number XM_001067836.1	5' TCCACAGGAGGATCTGGTTC 3'
Hypoxanthine phosphoribosyltransferase 1 (Hprt1)	5' GACCGGTTCTGTCATGTCG 3'
GenBank® Accession Number NM_012583.2	5' ACCTGGTTCATCATCACTAATCAC 3'
Vasa (DDX4)	5' GGAGGGAGGCCCAGGGAACC 3'
GenBank® Accession Number NM_001077647.1	5' GCCGCCTCGCTTGGAAAACC 3'
C-kit (CD117)	5' GGCTGACGTGCACTGACCCC 3'
GenBank® Accession Number NM_022264.1	5' AGCGGACCAGTGCGTCGTTG 3'
Deleted in azoospermia 1 (Daz1)	5' GCGCGGGCGCCGACGAAATCGGGA 3'
GenBank® Accession Number NM_001025742.1	5' TGGCTCCTGGCTGTCCTGGAGCT 3'
Thiosulfate sulfurtransferase SSEA1 (rhodanese)	5' ATTAAAGCTGCCAAATTGATTC 3'
GenBank® Accession Number XP_342833.2	5' GACTACAGATGAGAACCTCCCA 3'
Stage-specific embryonic antigen-3 (SSEA-3)	5' CAGCATGAATTCTTTCAGAGAACTCC 3'
synthase (b3GalT-V)	5' GCAAGAGGATCCCAGATCGTCACAAA 3'
GenBank® Accession Number NM_001122993.1	
CD29 (integrin beta-1)	5' GACCCCTCCGAGAGGCGGAA 3'
GenBank® Accession Number NM_017022.2	5' TGGCCGGAGCTTCTCTGCCAT 3'

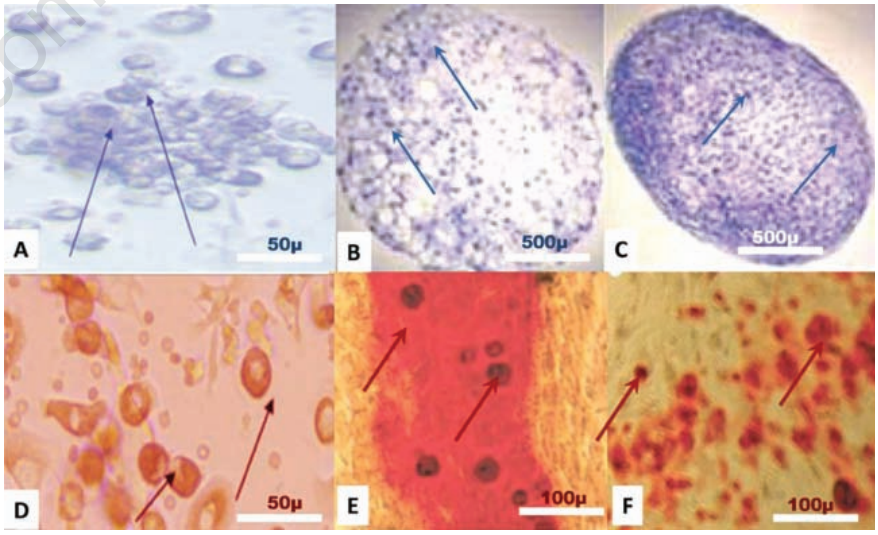

Figure 2. Morphological and histological staining of differentiated bone marrow mesecnchymal stem cells (BM-MSCs) into chondrocytes that were stained with Alcian blue stain. The matrix secreted by these cells was stained blue. Right arrows pointed to the matrix and left arrows pointed to chondrocytes (Figure 2A, 2B and 2C). Morphological and histological staining of differentiated BM-MSCs into osteoblasts that were with Alizarin red stain. The calcified matrices secreted were stained positive by alizarin red (Figure 2D, 2E and 2F). Right arrows pointed to the calcified matrices and left arrows pointed to osteoblasts (Figure 2D and 2F), whereas, left arrow pointed to the matrix and right arrow pointed to an osteoblast in Figure 2E.

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germ cells, Sertoli cells, and Leydig cells. Moreover, Hua *et al.*[7] reported that human fetal MSCs differentiates into spermatids. This finding raises the possibility that BMC might be utilized in the treatment of male infertility and testosterone deficiency.[21]

Up to our to our knowledge, this is the first time to confirm the potential use of transdifferentiated MSCs in experimental azoospermia in rats through quantitative gene expression assessment of premeiotic and postmeiotic markers in rat testicular tissue 12 weeks after MSCs transplantation. The molecular mechanism underlying adult stem cell plasticity and the targeting of MSCs differentiation toward germ cells remain unclear.[22] Testicular tissue represents a unique microenvironment for donor stem cell migration, proliferation, differentiation. The testes are protected from immunological influences by the blood–testis barrier, thereby allowing the recipient to host donor cells without rejection. This fact was demonstrated in the present study by the detection of fluorescent-labeled MSCs in rat testicular tissue 12 weeks after transplantation.

It has been well established that RA is deeply involved in the regulation of testicular function. An increase or a decrease in levels of RA could lead to spermatogenetic disorders or decrease in spermatogenesis. In a recent study, it was suggested that the testicular environment may control and support the differentiation of MSCs into somatic and germ cells. In our study, rat MSCs can be differentiated into germ cells *in vitro* by RA treatment and in the seminiferous tubules of the recipient rat *in vivo*. These findings were evidence by the detection of gene expression of a primordial germ cell marker (VASA), specific molecular markers of germ cells (c-kit, Daz1) *in vitro* and by the *in vivo* detection of gene expression of the premeiotic marker (Daz1) and post-meiotic markers (c-kit, Stra 8) in the testicular tissues of group 4 rats that received differentiated MSCs. Interestingly, these results are consistent with former reported results with mammalian BMCs.[21-25]

These findings were also reported by Nayernia *et al.*[15] , Check *et al.*[26] and Anserini *et al.*[27] Jacob *et al.*[21] reported recovery of spermatogenesis in 14% of men who had undergone autologous, allogenic bone marrow or peripheral stem cell transplant after either chemotherapy or total body irradiation regimens.

Huang *et al.*[28] stated that human umbilical cord Wharton's jelly-derived mesenchymal stem cells (HUMSCs) could form germ cells *in vitro* in all-trans retinoic acid, testosterone and testicular-cell-conditioned medium. HUMSCs showed expression of germ-cell-specific markers Oct4 (POUF5), c-kit, CD49f (a6), Stella (DDPA3), and Vasa (DDX4).

OCT4, SSEA-1 and SSEA-3 are stem cell markers that regulate pluripotency and are detected in both pluripotent cells and early

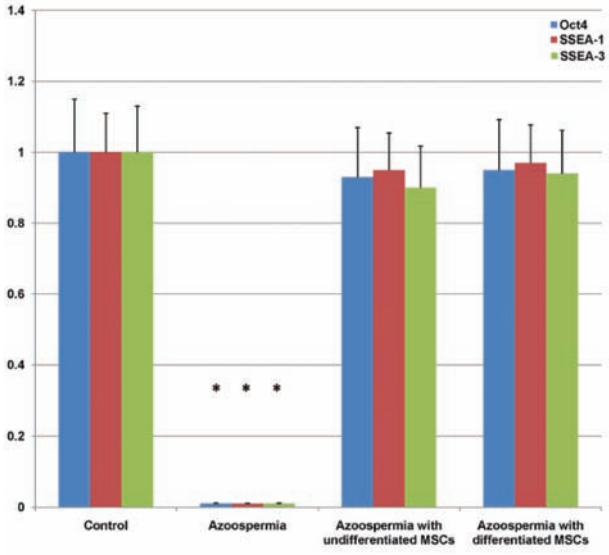

Figure 3. Relative gene expression of Oct4, SSEA-1, SSEA-3 in the studied rat groups. Genes were detected 12 weeks after mesenchymal stem cells transplantation.

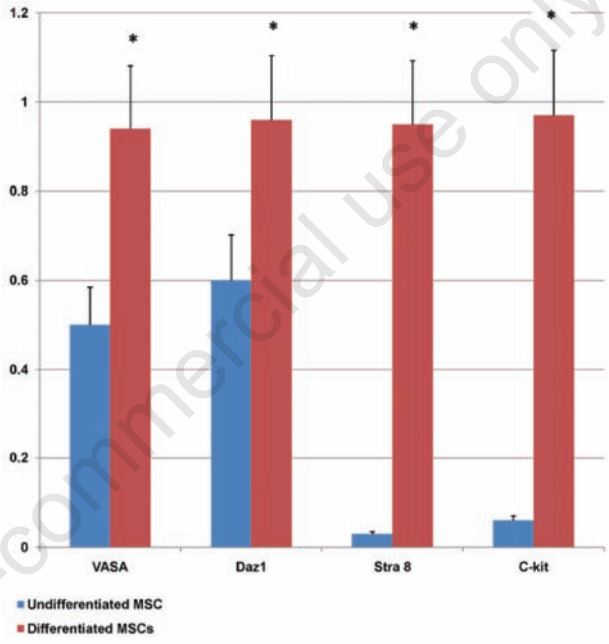

Figure 4. Relative gene expression of VASA, c-kit, Daz1 in undifferentiated mesenchymal stem cells (MSCs) and in the *in vitro* differentiated MSCs. Genes were detected 2 weeks after *in vitro* trans-differentiation of MSCs.

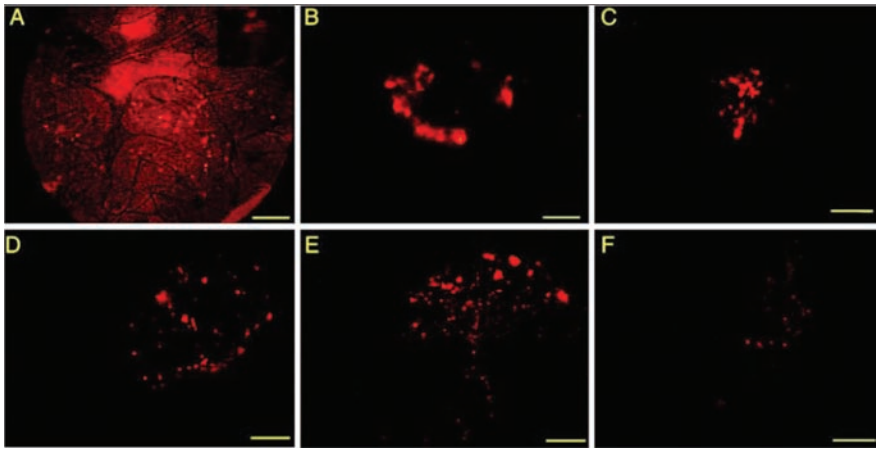

Figure 5. PKH26-fluorescent labelled stem cells in the seminiferous tubules of recipient rats. Mesenchymal stem cells (MSCs) labeled with the PKH26 showed strong red autofluorescence after transplantation into rats, confirming that these cells were seeded into the seminiferous tubules. A, F bars = 200 μm; B–E bars = 50 μm.

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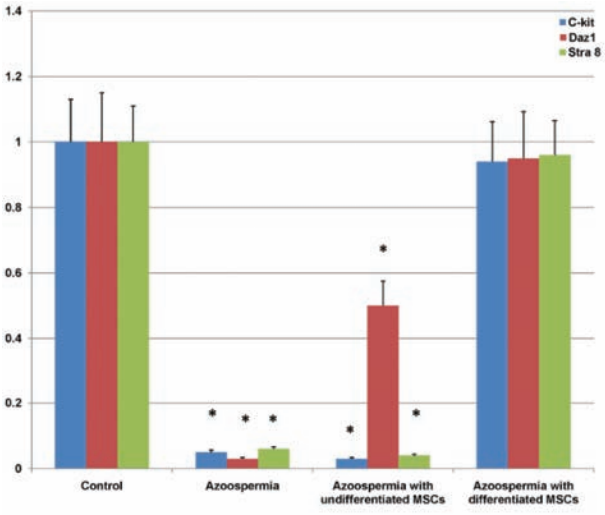

Figure 6. Relative gene expression of Daz1, C-kit, Stra 8 in the studied rat groups; *in vivo* study. Genes were detected 12 weeks after mesenchymal stem cells (MSCs) transplantation.

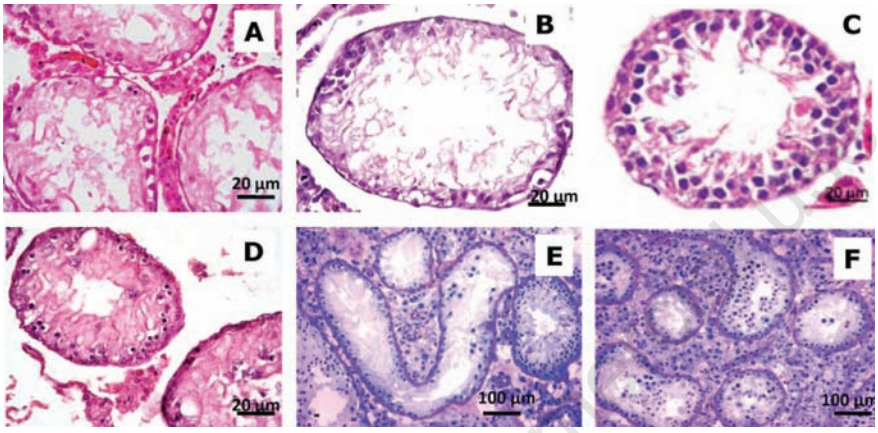

Figure 7. Busulfan treated testicular tissues. A, B) Shredded degenerated spermatogenic cells with appearance of some sertoli cells. C) Appearance of some spermatogenic cells. D-F) Shredded damaged spermatogenic cells with some sertoli cells.

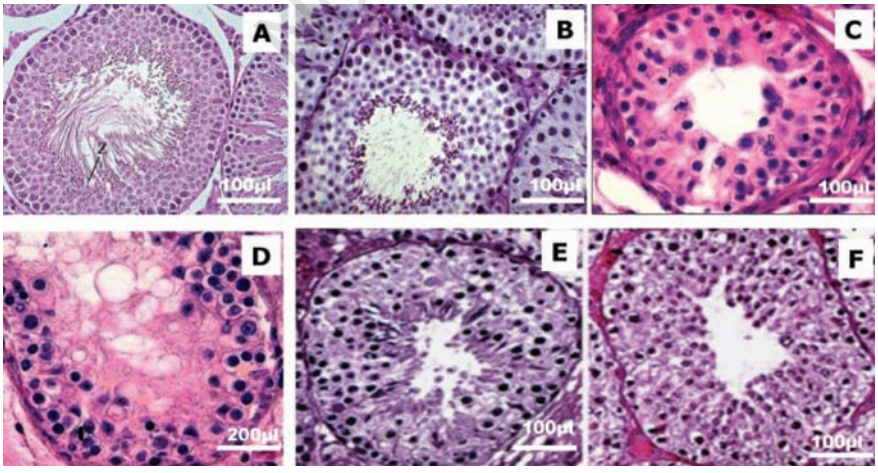

Figure 8. A) Control rats. B, C) Azoospermic rats that received differentiated mesenchymal stem cells (MSCs) showing primary, secondary spermatocytes and spermatids. D-F) Azoospermic rats that received undifferentiated MSCs showing some spermatogenic cells with no spermatids nor spermatozoa.

germ cells.[29] In the present study, these markers were detected in MSCs isolated from rat bone marrow. Levels of VASA are increased in germ cells such as PGC from the late migration stage to the post-meiotic stage.[8] Primordial germ cells as well as spermatogonia express c-kit; the cytokine receptor at a relatively high level. It has been proved that DAZL is a germ-cell-specific protein responsible for gametogenesis and contributes to the most common genetic cause of male infertility. DAZL is found to be the causal factor of azoospermia factor C. In the present study, we noted that BMC could differentiate into male germ cells after RA treatment *in vitro* and these cells expressed VASA. MSCs could also transdifferentiate into premeiotic and postmeiotic cells in the recipient's seminiferous tubules *in vivo* and these cells expressed DAZ1, c-kit and STRA8. Interestingly, these results are consistent with former reported results with mammalian BMCs.[16,21-25] Furthermore, 12 weeks after the transfer of BMCs into recipients' testes, labeled MSCs were detected in the seminiferous tubules of the recipient rats.

Recent studies have shown that germ cells can be generated successfully from somatic stem cells by incubating them in an artificial microenvironment simulating germ-cell development *in vivo*. This conditioning medium includes testicular-cell-conditioned medium, retinoic acid (RA) or testosterone. The testis is an abundant source of numerous growth factors such as bone morphogenetic protein 4, leukemia inhibitory factor, stem cell factor, and others which are needed for the development of germ cells.[30-33] RA, an active derivative of vitamin A, is required for the transition into meiosis for male germ cells and spermatogenesis.[34-36] Furthermore, testosterone promotes differentiation of ESCs and is essential for spermatogenesis *in vivo*.[37,38] These facts support our finding of the *in vivo* regeneration of spermatocytes and spermatids in the differentiated MSCs recipient rats.

In conclusion, MSCs have potentials for transdifferentiation into germ cells *in vitro* and transdifferentiation into spermatids and spermatocytes *in vivo* in testicular microenvironment.

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