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Differentiation of bone marrow derived mesenchymal stem cells into male germ-like cells in co-culture with testicular cells

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Objective. Stem cell therapy, specifically, pre-induction of mesenchymal stem cells toward male germ-like cells may be useful in patients with azoospermia. The aim of this study was to evaluate *in vitro* differentiation of mouse bone marrow-derived mesenchymal stem cells (BMSCs) into male germ-like cells by indirect co-culture with testicular cells in the presence of bone morphogenetic protein 4 (BMP4).

Methods. Experimental groups included: control (mouse BMSCs), treatment group-1 (BMSCs treated with BMP4), treatment group-2 (indirect co-culture of BMSCs with mouse testicular cells in the presence of BMP4) and treatment group-3 (indirect co-culture of BMSCs with testicular cells). BMSCs-derived male germ-like cells were evaluated by the expression of *Dazl*, and *Stra8* using RT-qPCR.

Results. Stra8 gene expression was significantly increased in the treatment group-2 and Dazl gene was significantly increased in the treatment group-1 compared to other groups. In conclusion, indirect co-culturing of BMSCs with testicular cells and BMP4 leads to the differentiation of BMSCs into male germ-like cells which express specific male germ-like genes. Testicular cells released factors that contributed to the differentiation of BMSCs into male germ progenitor cells.

Conclusion. This study suggests that mesenchymal stem cells may be differentiated into male germ-like cells and therefore, may be a novel treatment option for men with azoospermia.

Key words: BMSCs, Bone Morphogenetic Protein 4, indirect co-culture, *Stra8*, *Dazl*

Infertility is defined as failure to achieve pregnancy after one year of regular unprotected sexual intercourse (Singh et al. 2016). Infertility in men may be caused by incomplete spermatogenesis, pituitary gland disorders, testicular cancer, aplasia of germ cells, varicoceles, environmental factors, congenital abnormalities, immune system disorders, and neurological factors (Iammarrone et al. 2003). There are two types of azoospermia: obstructive and non-obstructive. In the obstructive azoospermia, sperm release is blocked from the ejaculatory ducts, meanwhile in non-obstructive azoospermia, there is low levels of sperm in the semen (Hussein 2013). In recent years, several studies have focused on the stem cell differentiation towards germ like cells for the treatment of non-obstructive azoospermia (Shirazi et al. 2012; Miryounesi et al. 2013; Makoolati et al. 2016). The use of mesenchymal stem cells may be an appropriate approach for the treatment of azoospermia (Gimble et al. 2007).

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Bone morphogenetic protein 4 (BMP4) is a member family of transforming growth factor beta (TGF-β) (de Rooij 2001; West et al. 2009). At the cell surface of epiblast cells, BMP4 bind ALK3 and BMPR-II. BMP4 receptors consisting heteromeric complexes. The BMPR-II receptor phosphorylates the ALK3 receptor, BMP4 receptor regulated phosphorylation Smads (Smad1, Smad5, or Smad8). As soon as the activation, the Smads bind to Smad4 and are transmissed from the cytoplasm to the nucleus, they regulate the transcription of BMP4 target genes such as *Fragilis* gene (Saitou et al. 2003; de Sousa Lopes et al. 2004; Surani et al. 2004). Following this, cells lose their ability to respond to BMP4 and express *Stella*. At this stage, cells are called primordial germ cells (PGCs) expressing *c-Kit* (Pellegrini et al. 2003).

In the next stages of the differentiation, the cells express pre-meiotic (*Stra8*) and meiotic (*Sycp3*) genes (Shirazi et al. 2012). *Dazl* is introduced as a specific post-migratory marker of PGCs and mutations in this gene can cause azoospermia (Foresta et al. 2001; Shirazi et al. 2012). *Stra8* and *Piwil2* are specific markers for spermatogonial stem cells (SSCs) (Lee et al. 2006; Zhou et al. 2008).

Sertoli cells are chief somatic cells in the testis and are necessary for the spermatogenesis process because of their major role in the interactions with germ cells and facilitation of necessary nutrient transfer between the two (Skinner 1991). Additionally, Sertoli cells contribute to the development of the germ cells via follicle-stimulating hormone (FSH) and its receptor (Hansson et al. 1975). Other important cells in testis are myoid cells, which regulate the function of Sertoli cells through secretion of PmodS factor (Skinner and Fritz 1985). In addition, Leydig cells are testosterone producing cells, which interact with Sertoli cells to maintain spermatogenesis (Margioris et al. 1983; Skinner 1991). Leydig cells release several peptides that regulate Sertoli cells including β-endorphins, α-melanocyte stimulating hormone, and adrenocorticotropic hormone (Fabbri et al. 1988).

In this study, the effect of testicular cells, as optimum micronutrient, on the differentiation of BMSCs to male germ-like cells using *in vitro* co-culturing of mouse BMSCs with mouse testicular cells was investigated. BMP4 was used to induce the differentiation process of the BMSCs toward germ-like cells.

Materials and methods

BMSCs isolation and culture. BMSCs were collected from the femur and tibia of 4–6 weeks old NMRI mice by flushing with high glucose DMEM (Dulbecco's Modified Eagle's Medium; Gibco, Paisley, UK) containing 1% penicillin/streptomycin (Gibco, Paisley, UK). Fetal bovine serum (FBS) (20%) (Gibco, Paisley, UK) was also used for BMSCs culture. Cells were incubated for 48 h and non-adherent cells were removed by changing the medium after 2 days. The medium was changed every two days and continued until the third passage. Mesenchymal (CD90, CD44, and CD73) and hematopoietic (CD34, CD45) cell surface markers were used for identification of the BMSCs by flow cytometry. Briefly, BMSCs, at passage 3, were dissociated and the cell suspension was stained with fluorescence conjugated antibodies phycoerythrin-conjugated rat anti-mouse CD73, phycoerythrin-conjugated rat anti-mouse CD90, fluorescein isothiocyanate-conjugated rat anti-mouse CD44, phycoerythrin-conjugated rat anti-mouse CD34, fluorescein isothiocyanate-conjugated rat anti-mouse CD45 and (Abcam, USA) for 45 min at 4 °C. After washing with PBS, staining buffer was added and the cells were used for flow-cytometry analysis. Cells were incubated by isotype control anti-bodies to measure the nonspecific background signal. Flow-cytometry analysis was performed using the BD FACsort device (BD Biosciences, USA).

Isolation and preparation of testicular cells. Testicular cells were isolated from 2–3-day-old NMRI male mice. Mice were sacrificed by cervical dislocation and then two incisions were performed in the skin of the lower part of the abdomen. The testicles were separated and placed in Petri dishes containing PBS. The testicles were then dissociated by sharp-tip scissors and digested enzymatically using trypsin-EDTA (Gibco, Paisley, UK). After additional vigorous pipetting, the relatively homogenous solution was centrifuged at 2500 g for 10 min, the supernatant was removed and enzyme was neutralized by FBS. This method is in accordance with Lacham-Kaplan et al. (2006).

BMSCs co-culture and BMP4 treatment. BMP4 (10 ng/ml; Sigma-Aldrich) treatment and indirect co-culture for 7 days, was used for BMSCs differentiation toward male germ-like cells (Dudley et al. 2007; Hamidabadi et al. 2011; Yang et al. 2016). BMSCs of third passage were placed at the bottom of the culture plate and the testicular cells were placed on the 0.4μm pore diameter biological barrier (ThinCert™ cell culture insert for 24-well plates, Greiner Bio-One International, Australia). Cells were incubated for 7 days in DMEM medium supplemented with 2–5% fetal bovine serum added to BMP4.

RT-qPCR analysis. Real-time qPCR assay was used for *Dazl*, *Stra8*, and *Piwil2* mRNA expression analysis and the fold changes between the treatments relative

to control groups determined. All gene expression values were normalized to β-Actin gene as internal control. Total RNA was isolated using TRIZOL reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Isolated RNA was treated with 1uL DNase I enzyme according to the manufacturer's instructions (Fermentas, Germany). After that, 25 ng of purified RNA was used for synthesizing 25 µl cDNA using RevertAid™ First strand cDNA synthesis kit (Fermentas, Germany) according to the manufacturer's instructions. To perform real-time PCR, cDNA (25 ng of RNA) was used to quantify *Dazl*, *Stra8*, *and Piwil2* mRNA levels. All primers are listed in Table 1. The PCR reaction was synthesized in a 12.5 µl volume (sense and anti-sense primers, cDNA, SYBR Green,) and carried out for 40 cycles (Applied Biosystems). The delta CT method was used to analyze relative changes in mRNA levels.

Statistical analysis. In this experimental study, the quantification was performed using SPSS15 software based on three independent experiments. The results were presented as a mean ± SEM. Normal distribution of data was determined using the Kolmogorov Smirnov test and values of $p>0.05$ was considered normal. After testing for normality one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple group comparison test was performed. Values of $p\leq0.05$ were considered statistically significant.

Table 1
Primer sequences and PCR parameters. Primers for amplification of target sequences, their Gen Bank accession number and the size of the fragment amplified are presented

Gene	Accession no.	Sense 5 → 3 Anti-sense 5 → 3	Size (bp)
B-actin	NM_007393.5	GGTCATCACTATTGGCAACG ACGGATGTCAACGTCACACT	72
Dazl	XM_006523583.3	AAGGCAAAATCATGCCAAAC TCCTGATTTCGGTTTCATCC	133
Stra8	XM_006505829.2	CTCCTCCTCCACTCTGTTG GCGGCAGAGACAATAGGAAG	135
Piwil2	XM_006519329.3	CCTCCAGCTCTGTCTCCAAC CCTTGCTTGACCAAAAGCTC	95

Primers were designed by Gene Runner 3.05 software (Product by: info@genfanavaran.com). Dazl (Deleted in Azoospermia Like), Stra8 (Stimulated by RA gene 8) and Piwil2 (Piwi-like homolog2).

Results

BMSCs culture. Primary cultured BMSC cells were round, floating and their nuclei were invisible 12 h after culture (Figure 1A). After 3–4 days, BMSCs developed into colonies (Figure 1B). After 7–8 days, BMSCs reached 70–80% confluency after which the first passage was performed (Figure 1C). The cells were subsequently passaged every 3–4 days. BMSCs were

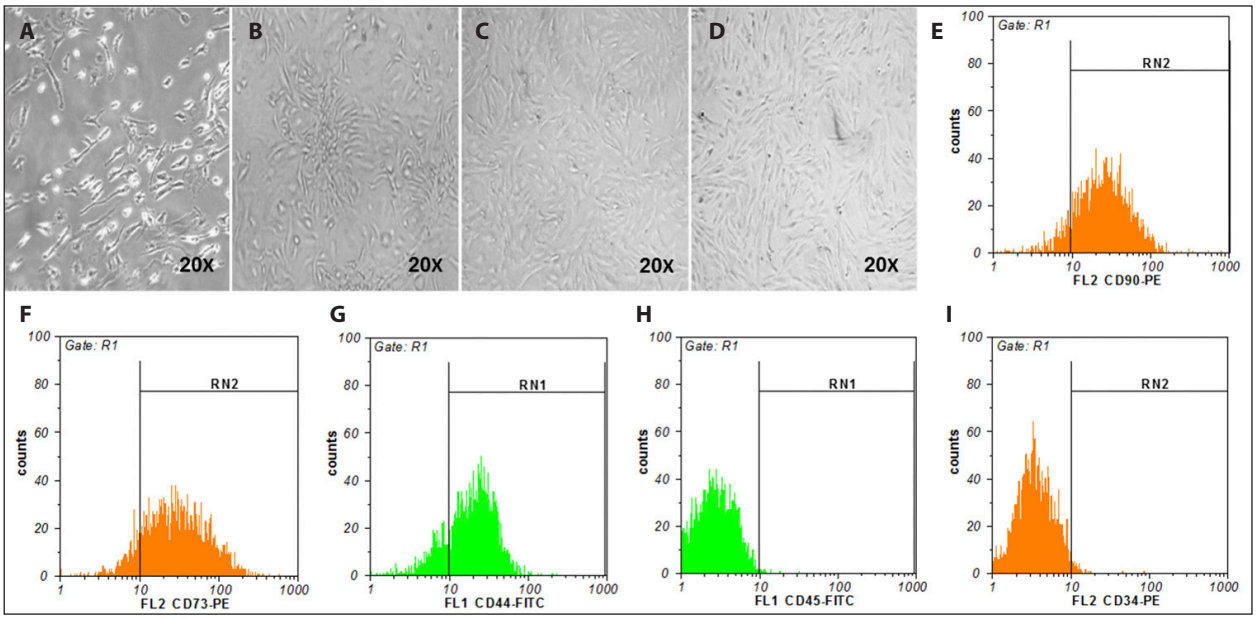

Figure 1. Representative photomicrographs of BMSCs isolation, culture and identification. **(A)** Primary culture after 12 h incubation; **(B)** cell colonies after 3–4 days of culture; **(C)** BMSCs after first passage; **(D)** homogenized BMSCs after 3 passages (day 14); **(E, F, G)** flow cytometric assay of CD90, CD73 and CD44, respectively (specific markers of mesenchymal stem cells); **(H, I)** flowcytometric assay of CD45 and CD34, respectively (markers for hematopoietic stem cells).

used for experiments at the third passage (Figure 1D). In order to identify BMSCs, flow cytometry analysis was used to detect specific mesenchymal stem cell markers. Isolated BMSCs were positive for specific mesenchymal cell surface markers including CD90 (87.48%), CD73 (85.86%) and CD44 (78.12%), and were negative for hematopoietic markers including CD45 (0.8%) and CD34 (2%) (Figure 1 E–I).

RT-qPCR results. Quantitative real time-PCR was used to detect the gene expression for specific germ-like cell markers *Dazl*, *Stra8*, and *Piwil2*. The control group (BMSCs without any treatment) was used as a calibrator and the expression of all genes were normalized to β-actin. The results are shown in Figure 2. The gene expression of *Dazl* in the BMSCs treated with BMP4 (3.73±0.26) and in the indirect co-culture of BMSCs with testicular cells (4.16±0.05) groups were increased significantly compared to the indirect co-culture of BMSCs with testicular cells in the presence of BMP4 group (0.46±0.33), respectively ($p<0.05$). *Piwil2* mRNA expression showed that its expression in groups of BMSCs treated with BMP4 (0.19±0.42), indirect co-culture of BMSCs with testicular cells (0.22±0.03) and indirect co-culture of BMSCs with testicular cells in the presence of BMP4 (0.03±0.00) were not significantly different compared to the control group, respectively. The expression level of *Stra8*, as a pre-meiotic gene, was increased significantly in the indirect co-culture of BMSCs with testicular cells in the presence of BMP4 group (6.18±0.78) compared with BMP4-treated and co-culture groups (3.08±0.29 and 2.01±0.28, respectively; $p<0.05$).

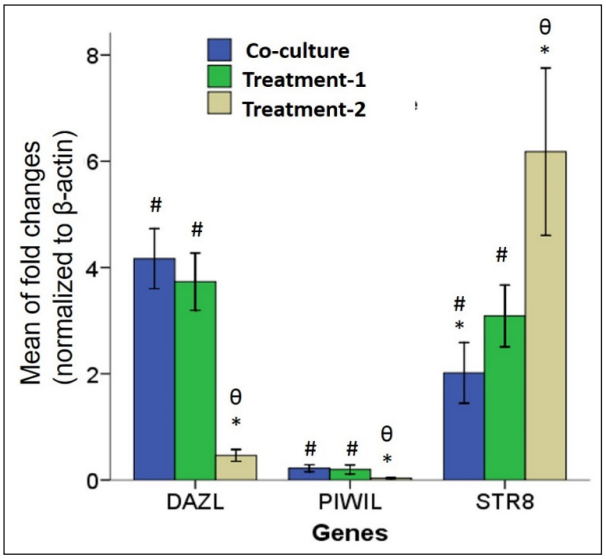

Figure 2. Quantitative real-time RT-PCR results of Dazl, Piwil2, and Stra8 genes relative to control group. mRNA is presented as the relative expression normalized to β-actin mRNA amplification. Control (BMSCs without any treatment); treatment-1 (BMSCs treated with BMP4); Treatment-2 (indirect co-culture of BMSCs with testis cells in the presence of BMP4) and co-culture (indirect co-culture of BMSCs with testis cells). The bars indicate the mean ± SEM; *$p<0.05$ vs. treatment-1group; $^{\theta}p<0.05$ vs. co-culture; $^{\#}p<0.05$ vs. treatment-2 group.

Discussion

The findings of this study indicate that indirect co-culturing of BMSCs with testicular cells in the presence of BMP4 induces BMSCs differentiation toward the male germ-like cells (MG-LCs) expressing *Dazl* and *Stra8* genes. Additionally, factors released from the testicular cells had an important role on the differentiation of BMSCs toward MG-LCs.

Clinically, mesenchymal stem cells are one of the best sources of stem cell therapy owing to their unique characteristics including their high rate proliferation and potential to differentiate into specific cells. Moreover, the cells can be harvested easily and are relatively safe for transplantation with no tumorigenicity making them attractive for using clinically (Gimble et al. 2007; Latifpour et al. 2014). In similar studies, Sertoli cells and biomolecules such as retinoic acid, testosterone, and bone morphogenetic proteins (BMPs) have been used as inducing factors for the differentiation of stem cells toward germ-like cells. In the present study, all the testicular cells were used as an effective micro-environment to derive germ-like cells from BMSCs. The Sertoli, Leydig and Myoid cells, as functional somatic testicular cells, can influence the spermatogenesis process and germ cell differentiation. Leydig, macrophage and myoid cells affect Sertoli cells, therefore they are in an indirect association with germ cell differentiation (Skinner 1991). Sertoli cells secrete metabolic biomolecules such as insulin-like growth factor (IGF) and transforming growth factor beta and alpha (TGF-β and α) that are needed for germ cell differentiation and the regulation of spermatogenesis (Walker and Cheng 2005). IGF, through specific receptors located on the surface of germ cells, regulates DNA synthesis (Vannelli et al. 1988).

BMP4 is produced by extra-embryonic ectoderm before and during gastrulation. BMP4 binding to R-Smad and Alk3 receptors on the surface of germ cells activates transcription factors leading to the expression of PGC specific genes (Saitou et al. 2002). Therefore, BMP4 signaling is effective in the formation and differentiation of PGCs. Collectively, testic-

ular cells are considered a potent source of many growth factors such as BMP4, leukemia inhibitory factor (LIF) and fibroblast growth factors (FGF-β), which are needed for spermatogenesis (Lacham-Kaplan et al. 2006). Several studies have shown that BMP4 is produced in the early stages of postnatal life by Sertoli cells and then its level decreases steadily. BMP4 receptors (Alk3 and BMPIIR) are expressed on spermatogonial cells mainly during the first week after birth (Pellegrini et al. 2003). In consideration of these events, we used testicular cells from 2–3-day old male mice. The microporous barrier of the insert filters used for cell culture in this study, only transfer soluble factors secreted by the testicular cells, which induced BMSCs to differentiate toward male germ-like cells.

Several studies have indicated that over-expression of specific genes related to germ cells differentiation (*Dazl*, *Stra8* and *Piwil2*) are important for *in vitro* induction of BMSCs into germ-like cells (Yu et al. 2009). The expression of *Dazl* also provided preliminary evidence of mitosis (type A and type B spermatogonia), meiosis (primary and secondary spermatocytes), and spermiogenesis (Silva et al. 2009). We demonstrated higher expression of *Dazl* in the treatment-1 group (BMSCs treated with BMP4) after 7 days of treatment, which confirmed the findings of other studies on the role of BMP4 on the differentiation of BMSCs to germ-like cells. Another study conducted by Shirazi et al. (2012) has shown stage specific embryonic antigen 1+ (SSEA1+) cells treated with BMP4 expressed specific molecular markers of PGCs such as *Oct4*, *fragilis*, *stella*, and *Mvh* (Shirazi et al. 2012). Our results indicate that the indirect co-culture differentiation of BMSCs for 7 days, resulted in significant up regulation of *Dazl*. Xie et al. (2015) have shown that umbilical cord mesenchymal stem cells co-cultured with Sertoli cells for 4, 7, and 14 days increase expression of *VASA*, *Dazl*, and *Stella* (Xie et al. 2015). Miryounesi et al. (2013) have reported that *Oct4*, *Dazl*, and *Stra8* are expressed in the germ cells derived from embryonic stem cells after 7 days co-culturing with Sertoli cells (Miryounesi et al. 2013). Our results are in concordance with these previous findings.

Silva et al. (2009) have treated embryonic stem cells with testosterone for 2, 4, 7, and 14 days and reported the expression of *Dppa3*, *Sycp3*, *Msy2*, *Stra8*, *Sycp1*, *Dazl*, *Prm1*, *Piwil2*, *Tex14*, *Akap3*, and *Odf2* in the cells, which were induced for 14 days (Silva et al. 2009). According to the findings of the above mentioned study, it can be concluded that testicular cells are more effective than Sertoli cells and testosterone hormones in the differentiation of stem cells into germ cells. Zhang et al. (2014) have shown that the *in vitro* co-culture of BMSCs with Sertoli cells followed by the transplantation of induced cells to testis of mice with azoospermia can lead to the expression of specific spermatogonial genes such as *VASA*, *Dazl*, and *c-Kit* (Zhang et al. 2014). In accordance with our findings, studies have demonstrated the role of testicular cells and their interactions with stem cells in the derivation of germ cells from stem cells (Geens et al. 2011). *Piwil2*, also known as the *Mili* gene, is a known molecular marker of spermatogonia and has functions in many processes including gametogenesis and spermatogonial stem cell self-renewal. *Piwil2* is expressed between 12–14 days post coitum (dpc) in mice (Lee et al. 2006). The data from the present study showed no significant increase in *Piwil2* mRNA expression during the 7 days co-culture treatment alone or in presence of BMP4 which suggests that longer periods of co-culturing may be needed for *Piwil2* to be expressed. *Stra8* is expressed when the germ cells pass the fetal stage from mitosis to meiosis. This change has been indicated in the mouse germ cells around 12.5 (dpc) (Zhou et al. 2008).

According to our knowledge, this is the first report demonstrating that BMP4 and indirect BMSCs co-culture with testicular cells may result in germ-like cells, which expressed *Dazl* and *Stra8*. In recent years, the production of PGCs has been challenged in different types of stem cells in animals. Several studies were performed in order to differentiate different kinds of stem cells, such as embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), bone marrow-derived mesenchymal stem cells (BMSCs) and umbilical cord stem cells (UCSCs) into germ cells. Makoolati et al. (2016) have demonstrated that in presence of BMP4 the embryonic stem cells can be induced into germ cells, which expressed β-Integrin, *Stra8* and *Mvh* genes (Makoolati et al. 2016). Latifpour et al. (2014) have shown that in the presence of BMP4 and retinoic acid human mesenchymal stem cells derived from umbilical cord can be induced into germ cell-like cells (Latifpour et al. 2014). Miryounesi et al. (2013) have shown the differentiation of embryonic stem cells into germ cells when co-cultured with Sertoli cells. The induced cells in their report express *Stra8*, *Sycp3* and *Dazl* genes after 7 days (Miryounesi et al. 2013). Additionally, Shirazi et al. (2012) have demonstrated that BMP4 can generate male germ-like cells from pluripotent bone marrow-derived SSEA-1+ cells (Shirazi et al. 2012).

It can be concluded that indirect co-culture of BMSCs with testicular cells in presence of BMP4 resulted in the derivation of germ-like cells. Our data may present a novel approach in the *in vitro* production of gametes and it use for the clinical applications.

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