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In vitro differentiation of bovine bone marrow-derived mesenchymal stem cells into male germ cells by exposure to exogenous bioactive factors

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Contents

Mesenchymal stem cells (MSC) are multipotent progenitor cells defined by their ability to self-renew and give rise to differentiated progeny. Previous studies have reported that MSC may be induced in vitro to develop into different types of specialized cells including male gametes. In vitro gamete derivation technology has potential applications as an alternative method for dissemination of elite animal genetics, production of transgenic animals and conservation of endangered species. This study aimed at investigating the in vitro effect of BMP4, TGFβ1 and RA on the potential for germ cell (GC) differentiation of bovine foetal MSC (bfMSC) derived from bone marrow (BM). The effect of BMP4, TGFβ1 and RA was analysed on the expression of pluripotent, GC and male GC markers on bfMSC during a 21-day culture period. bfMSC cultured under in vitro conditions expressed OCT4, NANOG and DAZL, but lacked expression of mRNA of VASA, STELLA, FRAGILIS, STRA8 and PIWIL2. Treatment with exogenous BMP4 and TGFβ1 induced a transient increase ($p < .05$) in DAZL and NANOG mRNA levels, respectively. However, exposure to RA was more effective in increasing ($p < .05$) expression of DAZL and regulating expression of OCT4 and mRNA levels of NANOG. These data suggest that bfMSC may possess potential for early GC differentiation, where OCT4, NANOG and specially DAZL may play significant roles in controlling progression along the GC lineage.

1 | INTRODUCTION

Mesenchymal stem cells (MSC) are a type of non-hematopoietic, adult stemlike cells that can be isolated from bone marrow (BM) and expanded in vitro. According to the international society for cellular therapy (ISCT), minimal criteria for defining human MSC cultures include adherence to plastic under standard culture conditions, expression of surface antigen markers CD105, CD73 and CD90, lack of expression of hematopoietic markers CD45, CD34 and CD14 and capacity for trilineage differentiation (Dominici et al., 2006). We have previously reported that MSC derived from bovine foetal BM (bfMSC) accomplish most of these criteria including plastic adherence, expression of CD73 and CD29, lack of expression of CD45 and CD34 and trilineage differentiation potential (Cortes et al., 2013; Dueñas et al., 2014). In addition to the differentiation potential, increasing amount of data indicate that MSC exert reparative functions through paracrine activity including immunomodulatory effects, and trophic functions involving antiapoptotic, angiogenic, mitogenic and antibacterial potentials (Caplan & Correa, 2011).

During the last few years, the in vitro derivation of germ cell (GC) lineages from stem cells has emerged as an exciting new strategy for obtaining mature gametes (West et al., 2013). Due to their nearly

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unlimited source and high differentiation potential, stem cells may be induced in vitro to develop into different types of specialized cells including male gametes, suggesting its potential applications to animal reproduction, as an alternative method for dissemination of elite animal genetics, production of transgenic animals and conservation of endangered species (Hill & Dobrinsky, 2006). Among adult stem cells, MSC may be suitable candidates for in vitro gamete derivation and subsequent use in cell transplantation due to: (i) plasticity that is not limited to mesodermal derivatives, (ii) availability of abundant sources of tissues for isolation in several animal species including bovine, (iii) high proliferative potential, (iv) simple and inexpensive isolation from foetal and adult somatic tissues and (v) high potential for cell therapy including autologous or allogeneic transplantation due to reduced intrinsic teratogenic formation and immune rejection. Recent studies have explored the GC-specific gene expression profile in male and female rat MSC (Ghasemzadeh-Hasankolaei, Eslaminejad, Batavani, & Ghasemzadeh-Hasankolaei, 2015) and evaluated the transdifferentiation potential of MSC derived from mice amniotic membrane (Afsartala et al., 2016), sheep bone marrow (Ghasemzadeh-Hasankolaei, Eslaminejad, Batavani, & Sedighi-Gilani, 2014), and human umbilical cord Wharton's jelly (Huang et al., 2010) and bone marrow (Hua, Yu, et al., 2009). These studies have generated important evidence that supports the potential capacity of MSC for differentiation into male GC lineage.

Gamete differentiation during embryogenesis is a well-orchestrated and highly complex process that involves sequential activation of several factors associated with cell proliferation and differentiation. In domestic animals, male GC are derived from a population of primordial germ cells (PGC) originated in the proximal epiblast (Lawson & Hage, 1994). PGC specification is induced by extrinsic factors secreted by extraembryonic ectoderm, including component of the Sma and Mad-related family (SMAD) signalling pathway, bone morphogenetic protein 4 (BMP4) and transforming growth factor β1 (TGFβ1) (Surani et al., 2004). After migration into the developing testis, PGC enter mitotic arrest and are reactivated after birth to initiate spermatogenesis. Recent studies have reported that decision of meiotic entry or mitotic arrest of post-migratory PGC is regulated by retinoic acid (RA) (Koubova et al., 2006). Male PGC do not enter meiosis because the enzyme CYP26b1 expressed in somatic cells in the male genital ridge degrades RA. Activity of RA is exerted inside the nucleus during gametogenesis through their binding to RA receptors (RAR) in Sertoli cells (RARα), round spermatids (RARβ) and type A spermatogonia (RARγ) (Vernet et al., 2006). RA favours spermatogonial differentiation through a direct action and an indirect effect mediated by BMP4 secreted by Sertoli cells (Pellegrini, Grimaldi, Rossi, Geremia, & Dolci, 2003).

Taking into account the previous information, bfMSC are promising cell sources for the derivation of gametes in animal species not studied so far, including bovine, giving the opportunity for development and future applications of new reproductive technologies in animals. This study aimed at investigating the in vitro effect of BMP4, TGFβ1 and RA on the potential for GC differentiation of bfMSC derived from BM.

2 | MATERIALS AND METHODS

2.1 | Isolation and culture of bfMSC

All procedures have been approved by the Bioethical Committee of the National Commission for Scientific and Technology Research from Chile (Fondecyt). Testes samples (n = 3) were collected from each of three slaughtered adult bulls and used for experiments as positive controls. bfMSC were isolated following a previously reported protocol that ensured establishment of MSC cultures that fulfil the minimal criteria for definition of MSC (Cortes et al., 2013; Dueñas et al., 2014). BM was aspirated from male bovine foetuses (n = 9; 7–8 months of gestation) collected at a local abattoir. The BM was drawn from femoral cavity into syringes containing high glucose (4.5 g/L) Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Islands, NY, USA) supplemented with 1000 IU heparin, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B. BM samples from three foetuses were pooled and washed twice with phosphate-buffered saline (PBS) and twice with DMEM. Then, cells were plated in DMEM (high glucose) supplemented with 10% foetal bovine serum (FBS; Gibco, Waltham, MA, USA), 100 IU/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B. Cells were incubated at 38°C in a humidified atmosphere containing 5% CO_2. After 2 days, non-adherent cells were removed by changing the culture medium. Following the initial 2 days, the medium was changed every two to three days. After three to four passages, cells were gently harvested when 90% confluent using 0.05% trypsin in 0.02% EDTA. Following determination of cell viability, cells were used to initiate experiments.

2.2 | Germ cell differentiation protocols

The effects of various concentrations of BMP4 (Cat. #314-BP; R&D systems, Minneapolis, MN, USA), TGFβ1 (Cat. #240-B; R&D systems, Minneapolis, MN, USA) or RA (Cat. #R2625; Sigma, Saint Louis, MO, USA) were first analysed on GC differentiation of bfMSC. Concentration–response experiments were performed using concentrations of 10, 50 and 100 ng/ml of BMP4; 1, 10 and 100 ng/ml of TGFβ1; and 0.01, 0.1 and 1 μM of RA in bfMSC cultures during 21 days. Cells (5×10^3/cm^2) isolated from three pools of BM collected from three male foetuses were seeded in T-25 culture dishes either in control or differentiation (three replicates each) medium and cultured for a 21-day experiment with the medium being changed every 2 days. Control medium for BMP4 and TGFβ1 experiments consisted of DMEM (high glucose) supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. Control medium for RA experiments consisted of DMEM (high glucose) supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B and DMSO (vehicle). Time–response experiments were performed using one selected concentration of 100 ng/ml of BMP4, 100 ng/ml of TGFβ1 or 0.1 μM of RA. Cell culture samples were taken on Days 0 and 21 (concentration–response) or on Days 0, 7, 14 and 21 (time–response).

2.3 | RNA extraction and cDNA synthesis

Approximately 1 × 10^5 bfMSC and testis samples were collected and immediately fixed in lysis buffer (Thermo Scientific, Waltham, MA, USA). Total RNA was extracted using GeneJET RNA purification kit (Thermo Scientific) according to the manufacturer's instructions. Total RNA was eluted in 50 μL of RNase-free water. The concentration and purity of the RNA in each sample were determined using Qubit RNA assay kit (Life Technologies, Waltham, MA, USA), and genomic DNA was removed using DNase I and RNase-free (Thermo Scientific). Samples were subjected to RT-PCR using a Brilliant II SYBR Green QRT-PCR kit AffinityScript Master Mix, 2-step kit (Agilent Technologies, Santa Clara, CA, USA). The reaction protocol consisted of incubation for 5 min at 25°C, 15 min at 42°C, 5 min at 95°C and hold at 4°C using a TC1000-G gradient thermocycler (SciLogex, Rocky Hill, CT, USA).

2.4 | Quantitative-PCR

Samples were analysed for expression of housekeeping β-ACTIN and GAPDH, pluripotent genes OCT4 and NANOG, GC genes FRAGILIS, STELLA and VASA, male GC genes DAZL, PIWIL2 and STRA8, and meiotic marker SCP3 expression by quantitative-PCR (Q-PCR) (Table 1). The β-ACTIN and GAPDH were selected as housekeeping genes based on previous analyses from our laboratory that demonstrated high stability during MSC culture (Cortes et al., 2013; Dueñas et al., 2014). Real-time PCR primers were designed using PrimerExpress software (Applied Biosystems Incorporated, Foster City, CA, USA; Table 1). Equivalence of amplification efficiencies among all primer-probe sets was confirmed using serial threefold dilutions of differentiated bfMSC cDNA. Each RT-PCR reaction (10 μL) contained the following: 2X Brilliant II SYBR Green QPCR master mix (5 μL), target forward primer (200 nM), target reverse primer (200 nM), cDNA synthesis reaction (1 μL) and nuclease-free PCR-grade water to adjust final volume. The PCR amplification was carried out in an Eco Real-Time PCR System (Illumina Incorporated, San Diego, CA, USA). Thermal cycling conditions were 95°C for 10 min, followed by 40 repetitive cycles at 95°C for 30 s, and 60°C for 1 min. The relative quantification of the target gene expression across treatments was evaluated using the comparative ΔΔCT method (Livak & Schmittgen, 2001). The CT value was determined by subtracting β-ACTIN CT value (most stable endogenous gene CT value) from the target CT value of the sample. Calculation of ΔΔCT involved using target gene expression (sample with the highest CT value or lowest target expression), including

TABLE 1 Sequence of primers used for Q-PCR analysis

Gene	Nucleotide sequence (5′–3′)	Accession number
Endogenous genes		
GAPDH	Forward CCTTCATTGACCTTCACTACATGG TCTA	NM_001034034.2
	Reverse TGGAAGATGGTGATGGCCTTTCCATTG	
β- ACTIN	Forward CGCACCACTGGCATTGTCAT	NM_173979.3
	Reverse TCCAAGGCGACGTAGCAGAG	
Pluripotency genes		
OCT4	Forward GAAAGAGAAAGCGGACGAG	NM_174580.2
	Reverse GTGAAAGGAGACCCAGCAG	
NANOG	Forward TAAGCACAGGGGGCAAAAGT	NM_001025344.1
	Reverse ATGGCTAAAAGGGGTGGAGG	
Germ cell genes		
FRAGILIS	Forward ATCTGCAGCGAGACCTCTGT	XM_002697323
	Reverse CCGATGGACATGATGATGAG	
STELLA	Forward TGCAAGTTGCCACTCAACTC	NM_00111110
	Reverse TCTTACCCCTCTCCGCCTAT	
VASA	Forward TGCTACTCCTGGAAGACTGA	JX_437185.1
	Reverse CGGTCTGCTGAACATCTCTA	
Male germ cell genes		
DAZL	Forward TCC AAG TTC ACC AGT TCA GG	NM_001081725.1
	Reverse CGT CTG TAT GCT TCT GTC CAC	
PIWIL2	Forward TCGTATTGATGATGTGGATTGG	XM_617223.3
	Reverse GGGAGCAGCAGGATTTCAC	
STRA8	Forward TGTGCCCAGGTGTTCATCTC	XM_015463130
	Reverse GGGGACTGTCACCTCATTGG	
Meiotic gene		
SCP3	Forward GCTGGAAAGATT TGGAGCTG	BC_102433
	Reverse ATCCCACTGCTGGAACAAAG	

FRAGILIS (Testis), Day 0 or untreated cells (bfMSC) as an arbitrary constant to subtract from all other CT sample values.

2.5 | Flow cytometry

The extent of GC differentiation was analysed at Days 0 and 21 of differentiation by determination of bfMSC population positive for Oct4, Nanog and Dazl using flow cytometry (FC). Approximately $1–3 \times 10^6$ cells were detached after incubation in EDTA for 10–20 min and then fixed with 4% paraformaldehyde in PBS for 10–15 min at 4°C. Samples were resuspended in a solution of 1% Triton X100 in PBS and incubated for 15 min at room temperature and then in 0.1% Tween in PBS for washing. Antigens were blocked using a solution of 3% bovine serum albumin (BSA) with 1.5 mg/ml of glycine during 1 h at room temperature. Then bfMSC were incubated overnight in primaries goat polyclonal anti-Oct4, goat polyclonal anti-Nanog antibodies (Cat. #sc-8628 and sc-30331; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit polyclonal anti-Dazl (Cat. #ab-34139; Abcam, Cambridge, MA, USA) diluted (1:50) in 3% BSA in PBS. Cells were washed thrice in 0.1% Tween in PBS and incubated in a solution of rabbit anti-goat igG conjugated with Alexa Fluor 488 (Cat. #A11078; Thermo Fisher Scientific, Rockford, IL, USA) or goat anti-rabbit igG conjugated with FITC (Cat. #ab-97050; Abcam) diluted (1:1000) in 3% BSA during 1 hr at room temperature. Then bfMSC were washed thrice in 0.1% Tween and then incubated with 3 μL propidium iodide for 5 min at room temperature. Cells were resuspended in IsoFlow buffer and analysed (three replicates) using a Gallios Flow Cytometer (Beckman Coulter, Brea, CA, USA) using a 488-nm laser light. The threshold for negative events was set on the first decade of fluorescence level histogram. Negative procedural control corresponded to cells not incubated with antibodies (autofluorescence) and cells incubated only with secondary antibody. Percentage of cells positive for autofluorescence and secondary antibody were subtracted from the percentage of cells positive with primary and secondary antibodies.

2.6 | Statistical analysis

Values of gene expression from three different replicates were transferred to a spreadsheet and then analysed using Infostat software (Cordoba, Argentina). Data were normalized to logarithmic scale in base 10 for normality, and mean values for each replicate were compared by one-way ANOVA. Gene expression values between days of culture and between treatments and controls were analysed using Duncan's multiple comparison test ($p < .05$).

3 | RESULTS

3.1 | Morphological characterization of bfMSC during germ cell differentiation

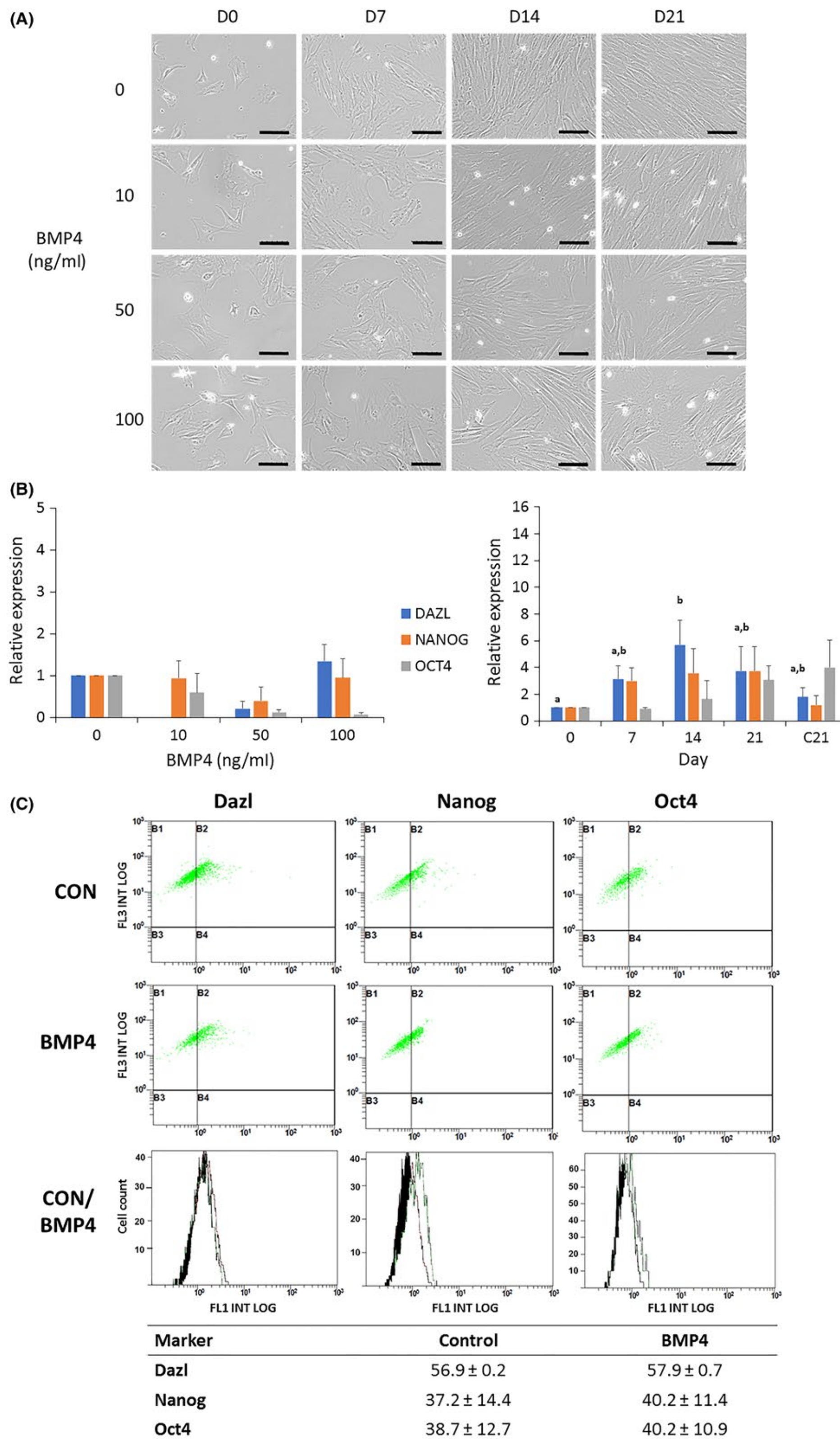
Isolation of bfMSC from BM was performed based on the capacity for plastic attachment under standard culture conditions that included DMEM media supplemented with 10% FBS. Colonies of fibroblast-like cells attached to the plastic were visualized at Days 5–6 after seeding. In order to evaluate the GC differentiation potential of bfMSC, concentration–response experiments were performed to determine the effect of RA, BMP4 or TGFβ1 that induced high GC marker expression and cell survival in bfMSC cultures. Thereafter, bfMSC were exposed to concentrations of 100 ng/ml BMP4, 100 ng/ml TGFβ1 and 0.1 μM RA during a 21-day culture period in order to analyse the temporal expression of pluripotent, GC and male GC genes during bfMSC differentiation. Treatment with increasing concentrations of BMP4 had a slight effect on bfMSC morphology and culture organization, including formation of cell projections and development of intricate cell interactions (Figure 1A). Similarly, treatment of bfMSC with 1 and 10 ng/ml of TGFβ1 induced changes in monolayer organization characterized by formation of scattered cell aggregates (Figure 2A). In comparison, exposure of bfMSC to 100 ng/ml of TGFβ1 induced formation of distinct and numerous cell aggregates. bfMSC cultured under the effect of increasing concentration of RA changed cell morphology, acquiring multiple cell projections and complex organization in monolayer cultures (Figure 3A).

3.2 | Levels of mRNA in bull testis samples and bfMSC during germ cell differentiation

Firstly, determination of the GC lineage was assayed by Q-PCR in tissue extracts from mature bull testis used as positive controls. All transcripts evaluated in bull testis, including pluripotent markers OCT4 and NANOG, GC genes FRAGILIS, VASA, STELLA, male GC genes DAZL, STRA8 and PIWIL2 and meiotic marker SCP3 were detected. Highest levels of mRNA in bull testis were detected for OCT4 (330-fold), VASA (27,753-fold), DAZL (123,341-fold) and PIWIL2 (8,292-fold) compared to FRAGILIS expression. Further analyses in bfMSC indicate that levels of mRNA of these genes are vastly greater in testes compared to bfMSC.

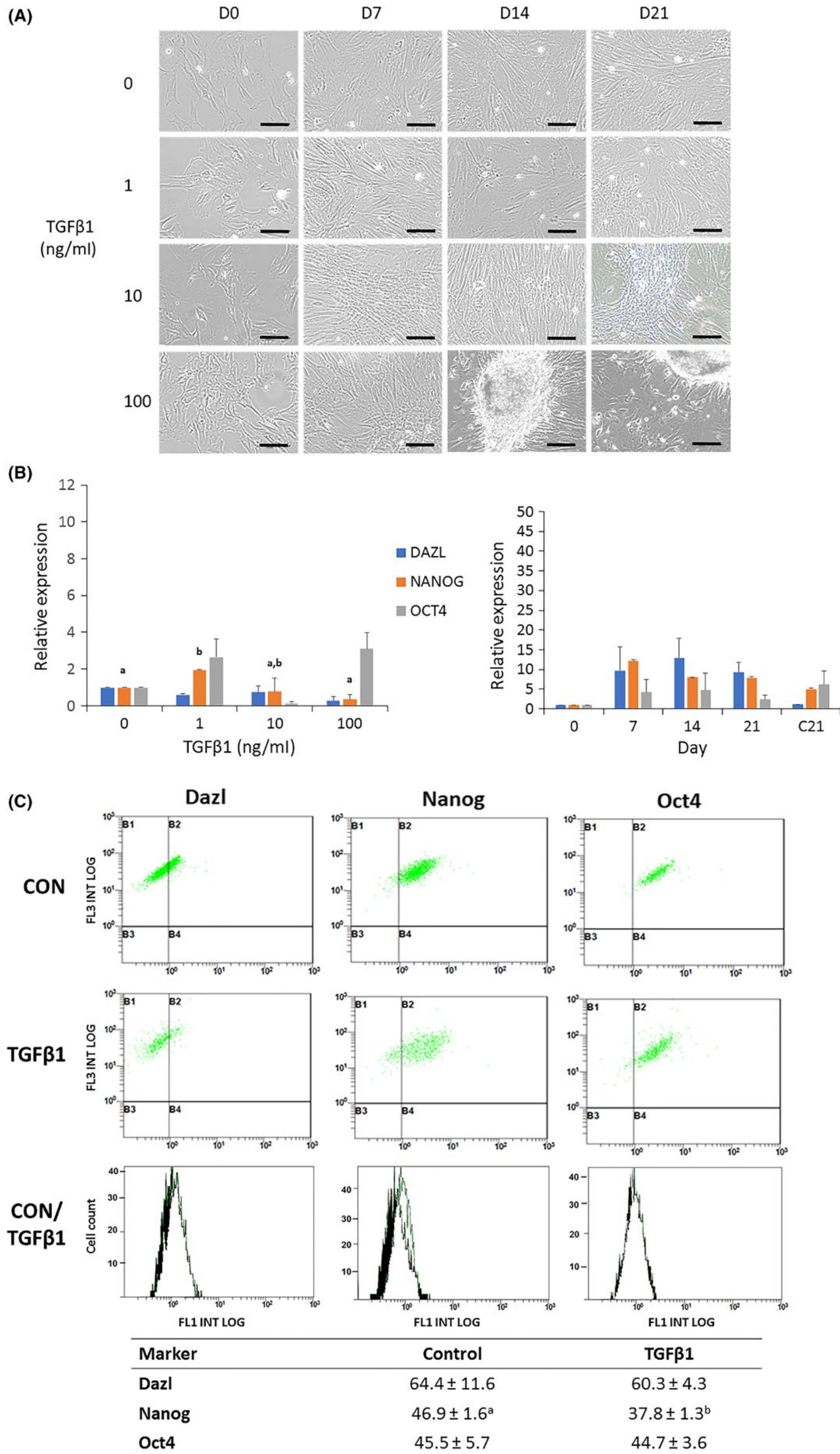
Levels of mRNA of DAZL, NANOG and OCT4 were not affected by BMP4 concentration (Figure 1B). However, temporal analyses detected up-regulation ($p < .05$) of DAZL mRNA levels at Day 14 of culture (5.7-fold Day 0). NANOG mRNA levels were higher ($p < .05$) in bfMSC treated with 1 ng/ml of TGFβ1 (1.9-fold Day 0) compared to untreated and bfMSC treated with 100 ng/ml (0.3- and 0.35-fold Day 0; Figure 2B). Levels of mRNA of DAZL, NANOG and OCT4 were not different ($p > .05$) between culture days in bfMSC exposed to 100 ng/ml of TGFβ1 during 21 days.

Levels of NANOG mRNA were increased in bfMSC treated with 0.1 μM (40.6-fold) and 1 μM (21.3-fold) of RA compared to untreated control (Figure 3B). When bfMSC were exposed to 0.1 μM of RA, mRNA levels of DAZL increased ($p < .05$) 6.5- and 7.9-fold, at Days 7 and 21, respectively. Moreover, bfMSC treated with 0.1 μM RA increased ($p < .05$) NANOG mRNA levels at Day 7, 14 and 21 compared to Day 0 (23.2-, 29.5- and 30.3-fold Day 0, respectively). Moreover, levels of mRNA of meiotic marker SCP3 in bfMSC exposed to BMP4, TGFβ1 and RA were not different ($p > .05$) between days of culture



Marker	Control	BMP4
Dazl	56.9 ± 0.2	57.9 ± 0.7
Nanog	37.2 ± 14.4	40.2 ± 11.4
Oct4	38.7 ± 12.7	40.2 ± 10.9

FIGURE 1 Germ cell in vitro differentiation of bfMSC under the effect of BMP4. (A) Treatment with variable concentrations of BMP4 (10, 50 and 100 ng/ml) had a slight effect on bfMSC morphology and culture organization. (B) Temporal analyses detected up-regulation ($p < .05$) of DAZL mRNA levels at Day 14 of culture. (C) Percentages of cells positive for Dazl, Nanog and Oct4 (white curve) were not affected ($p > .05$) by BMP4 treatment at Day 21 of culture (control, black curve). (a,b) Indicate significant ($p < .05$) difference compared to Day 0 (right graph). Scale bars: 500 µm. Abbreviations: CON, control; C21, negative control at Day 21 of differentiation; FL1/3 INT LOG, signal intensity of the protein expression in log scale



Marker	Control	TGFβ1
Dazl	64.4 ± 11.6	60.3 ± 4.3
Nanog	46.9 ± 1.6[a]	37.8 ± 1.3[b]
Oct4	45.5 ± 5.7	44.7 ± 3.6

FIGURE 2 Germ cell in vitro differentiation of bfMSC under the effect of TGFβ1. (A) Exposure of bfMSC to 100 ng/ml of TGFβ1 induced formation of numerous cell aggregates. (B) NANOG mRNA levels were higher ($p < .05$) in bfMSC treated with 1 ng/ml of TGFβ1 compared to untreated and bfMSC treated with 100 ng/ml. (C) Percentages of cells positive for Nanog (white curve) was reduced ($p < .05$) after exposure to TGFβ1 at Day 21 of culture (control, black curve). (a,b) Indicate significant ($p < .05$) difference compared to untreated cells. Scale bars: 500 μm. Abbreviations: CON, control; C21, negative control at Day 21 of differentiation; FL1/3 INT LOG, signal intensity of the protein expression in log scale

and treatments suggesting that treated bfMSC did not progressed into meiosis (Figure 4).

3.3 | Flow cytometric analyses of germ cell differentiated bfMSC cultures

Percentages of cells positive for Oct4, Nanog and Dazl proteins were not affected ($p > .05$) by BMP4 treatments at Day 21 of culture (Figure 1C). However, the percentages of cells positive for Nanog protein were reduced (37.8 ± 1.3 vs. 46.9 ± 1.6) after exposure to TGFβ1 at Day 21 of culture (Figure 2C). At Day 21, percentage of cells positive for Dazl protein was higher ($p < .05$) after treatment with RA (75.4 ± 8.5%) compared to untreated controls (57.5 ± 0.4; Figure 3C). Moreover, percentage of cells positive for Oct4 were lower ($p < .05$) after treatment with RA (83.2 ± 3.4%) compared to untreated control (99.4 ± 0.4; Figure 3C).

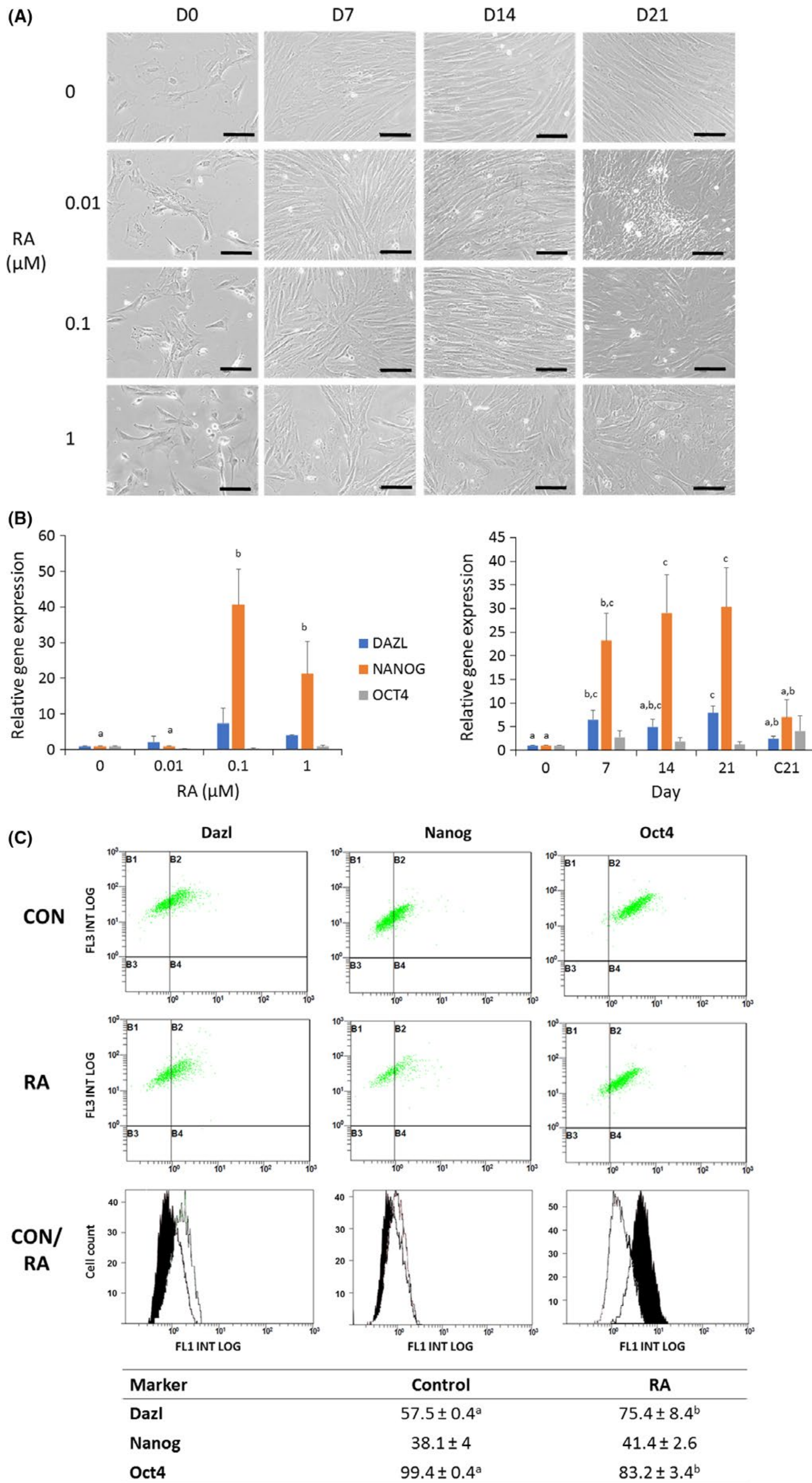
4 | DISCUSSION

Determination of the GC lineage was initially assayed in tissues extracts from mature bull testis used as positive controls. All transcripts evaluated in bull testis were detected. In comparison, bfMSC exposed to BMP4, TGFβ1 and RA during a 21-day culture period expressed OCT4, NANOG, DAZL and SCP3 but lacked expression of mRNA of VASA, STELLA, FRAGILLIS, STRA8 and PIWIL2. This pattern of expression suggests that bfMSC may possess potential for early GC differentiation, where OCT4, NANOG and DAZL may play significant roles in controlling progression along the GC lineage.

Supplementation of various doses of BMP4 had no effect on mRNA levels of OCT4, NANOG or DAZL gene and protein expression at Day 21 of culture. However, addition of 100 ng/ml of BMP4 increased DAZL mRNA levels in differentiating bfMSC at Day 14 of culture. BMP4 activity is mediated by receptor ALK3 and transducer SMAD5, exerting both mitogenic and differentiative effects (Pellegrini et al., 2003). The effect of BMP4 on GC differentiation has been previously characterized in ovine BM-MSC, where 100 ng/ml of BMP4 had no effect on mRNA levels of DAZL, VASA, PIWIL2 and OCT4 after 21 days of culture (Ghasemzadeh-Hasankolaei, Sedighi-Gilani, & Eslaminejad, 2014). However, a two-step culture method using 25 ng/ml of BMP4 for 5 days followed by 1 μM of RA for 12 days induced DAZL and STRA8 expression in mice MSC (Afsartala et al., 2016). Canine adipose tissue (AT) MSC, treated for 7 days with 12.5 ng/ml of BMP4, increased expression of PGC markers PRDM1, PRDM14 and male GC markers DMRT1 and PLZF but showed no effect on VASA expression (Wei et al., 2016). Overall, these results suggest that BMP4 regulation of GC gene expression may vary, according to concentration and time in culture medium. In the case of bfMSC, exposure to BMP4 only induced a transient increase in the expression of DAZL.

Despite the lack of effect in OCT4 and DAZL expression, supplementation of TGFβ1 induced a dose–response effect on NANOG mRNA levels with lower expression in bfMSC exposed to 100 ng/ml compared to 1 ng/ml. TGFβ1 is present in foetal and neonatal developing testis regulating cell proliferation, differentiation and playing a critical role in normal sexual competence (Ingman & Robertson, 2007). Although NANOG play a crucial role in maintenance of pluripotent state in embryos and derived cells in most mammalian species (Loh et al., 2006), the exact function of this transcriptional factor in adult stem cells has not been fully established. In previous studies, we reported that the multipotent capacity of bfMSC represented by NANOG gene expression is reduced according to the acquisition of hepatogenic phenotype (Dueñas et al., 2014). Thus, our data suggest that TGFβ1 treatment may result in loss of the multipotent state in bfMSC; however, the effect of TGFβ1 was not sufficient to initiate differentiation into the GC lineage.

In our study, supplementation of 0.1 μM RA induced up-regulation of DAZL expression and NANOG mRNA levels in bfMSC from Day 7 to Day 21 of culture. Moreover, Oct4 expression was down-regulated in bfMSC after 21-day culture in response to the same RA concentration. RA is a derivative from vitamin A that plays crucial roles in GC production, triggering GC meiosis and gametogenesis and influencing embryonic patterning and development (Koubova et al., 2006). Adult and foetal human BM-MSC exposed to 1 or 10 μM RA have been reported to increase expression of early GC markers OCT4, STELLA, NANOG and VASA, and male GC-specific markers including DAZL, TH2, c-KIT, STRA8 and SCP3 (Drusenheimer et al., 2007; Hua, Pan, et al., 2009). Previous reports in ovine MSC indicated that treatment with 10 μM of RA during a 21-day culture period down-regulated OCT4 levels but was unable to induce DAZL expression (Ghasemzadeh-Hasankolaei, Eslaminejad, et al., 2014). Thereafter, these authors reported that the same RA treatment increased mRNA levels of VASA, PIWIL2, OCT4 and DAZL; however, it was less effective to induce GC differentiation compared to TGFβ1 treatment (Ghasemzadeh-Hasankolaei, Eslaminejad, & Sedighi-Gilani, 2016). Conversely, in our study, treatment of bfMSC with RA was more effective in increasing expression of DAZL and regulating expression of OCT4 and mRNA levels of NANOG compared to TGFβ1. Differences in results between studies may be associated with



Marker	Control	RA
Dazl	57.5 ± 0.4[a]	75.4 ± 8.4[b]
Nanog	38.1 ± 4	41.4 ± 2.6
Oct4	99.4 ± 0.4[a]	83.2 ± 3.4[b]

FIGURE 3 Germ cell in vitro differentiation of bfMSC under the effect of RA. (A) bfMSC cultured in the presence of different concentrations of RA (0.01, 0.1, 1 μM) displayed progressive cell morphology changes during the 21-day experiment. (B) Levels of NANOG mRNA were increased ($p < .05$) in bfMSC treated with 0.1 and 1 μM of RA compared to untreated control. When bfMSC were exposed to 0.1 of RA, mRNA levels of DAZL and NANOG increased ($p < .05$) at Days 7 and 21 compared to Day 0, respectively. (C) Percentage of cells positive for Dazl (white curve) was higher ($p < .05$) and percentage of cells positive for Oct4 was lower ($p < .05$) after treatment with RA compared to untreated control (black curve). (a,b,c) Indicate significant ($p < .05$) difference compared to Day 0. Scale bars: 500 μm. Abbreviations: CON, control; C21, negative control at Day 21 of differentiation; FL1/3 INT LOG, signal intensity of the protein expression in log scale

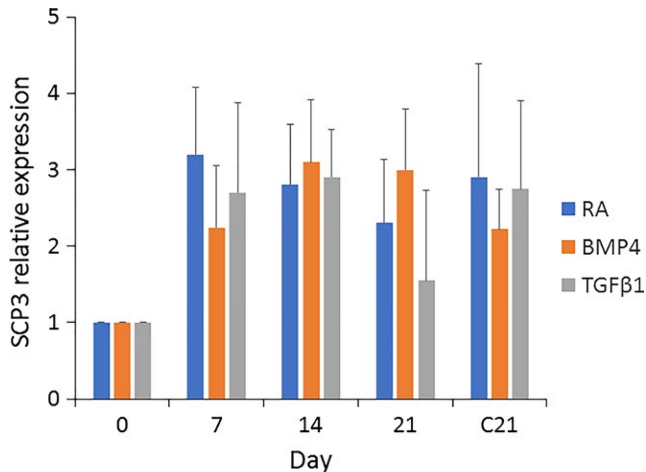


FIGURE 4 Levels of mRNA of meiotic marker SCP3 in differentiating bfMSC. Levels of mRNA of meiotic marker SCP3 in bfMSC exposed to BMP4, TGFβ1 and RA were not different ($p > .05$) between days of culture and treatments

multiple factors including experimental conditions and animal species source of MSC.

Increasing amount of data has demonstrated that DAZL is a master gene controlling GC differentiation. During migration of PGC into the undifferentiated gonads, DAZL is expressed playing essential roles in development of PGC and in differentiation and maturation of GC (Yen, 2004). The relevance of DAZL has been demonstrated in DAZL knockout mice, where embryos display reduced expression of GC-specific genes including STELLA and VASA and post-natal males present impairment in progression from A to A1 spermatogonia and meiotic arrest resulting in azoospermia and sterility (Lin & Page, 2005; Ruggiu et al., 1997). Considering the crucial role of DAZL in GC differentiation, future studies aiming at programming MSC along the GC lineage should attempt at increasing expression of DAZL to induce subsequent activation of early GC and male GC genes.

In conclusion, bfMSC cultured under in vitro conditions expressed pluripotent markers OCT4, NANOG and male GC gene DAZL, but lacked mRNA of VASA, STELLA, FRAGILLIS, STRA8 and PIWIL2. Treatment with exogenous BMP4 and TGFβ1 induced transient increase in DAZL and NANOG mRNA levels; however, exposure to RA was more effective in increasing expression of DAZL and regulating expression of OCT4 and mRNA levels of NANOG. These data suggest that bfMSC may possess potential for early GC differentiation, where OCT4, NANOG and specially DAZL may play significant roles in controlling progression along the GC lineage.

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CONFLICT OF INTEREST

None of the authors have any conflict of interests to declare.

AUTHOR CONTRIBUTIONS

Jahaira Cortez performed the experiments and collected and analysed the data. Javiera Bahamonde was involved in the acquisition and analysis of the data and participated in the development of the manuscript. Monica De los Reyes was involved in the experimental design and the drafting of the paper. Jaime Palomino processed the samples and analysed the data from the flow cytometric analysis. Cristian Torres participated in the experimental design, analysis of the data and drafting of the paper. Oscar Peralta was involved in the design the study, analysis of the data including statistical analysis and writing of the paper.

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