



Glial cell derived neurotrophic factor induces spermatogonial stem cell marker genes in chicken mesenchymal stem cells



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ABSTRACT

Mesenchymal stem cells (MSCs) are known with the potential of multi-lineage differentiation. Advances in differentiation technology have also resulted in the conversion of MSCs to other kinds of stem cells. MSCs are considered as a suitable source of cells for biotechnology purposes because they are abundant, easily accessible and well characterized cells. Nowadays small molecules are introduced as novel and efficient factors to differentiate stem cells. In this work, we examined the potential of glial cell derived neurotrophic factor (GDNF) for differentiating chicken MSCs toward spermatogonial stem cells. MSCs were isolated and characterized from chicken and cultured under treatment with all-trans retinoic acid (RA) or glial cell derived neurotrophic factor. Expression analysis of specific genes after 7 days of RA treatment, as examined by RT-PCR, proved positive for some germ cell markers such as *CVH*, *STRA8*, *PLZF* and some genes involved in spermatogonial stem cell maintenance like *BCL6b* and *c-KIT*. On the other hand, GDNF could additionally induce expression of *POU5F1*, and *NANOG* as well as other genes which were induced after RA treatment. These data illustrated that GDNF is relatively more effective in diverting chicken MSCs towards Spermatogonial stem cell –like cells in chickens and suggests GDNF as a new agent to obtain transgenic poultry, nevertheless, exploitability of these cells should be verified by more experiments.

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1. Introduction

Stem cells have the potential to differentiate into various cell types and could be categorized according to their differentiation potency. Spermatogonial stem cells (SSCs) have been considered as pluripotent stem cells because they are capable of differentiating into almost all cell types in the body (Kanatsu-Shinohara et al., 2004). SSCs have great applications in many fields including devel-

opmental biology, germ cell related disorders like male infertility, transgenic technologies in industrial animals like poultries and survival of rare or extinct species.

Thus a priority in stem cell research is to establish optimal conditions for derivation and maintenance of SSCs *in vitro*. Isolation and manipulation of these cells is difficult (Tegelenbosch and de Rooij, 1993; Hofmann, 2008). Although, several studies have reported culturing SSCs in the last few years but their maintenance remains a difficult task (Kanatsu-Shinohara et al., 2003; Momeni-Moghaddam et al., 2013).

Breakthroughs in stem cell research have shown that stem cells from one type could be transdifferentiated into another type. Several reports illustrated that mesenchymal stem cells could be differentiated into cells expressing the molecular markers of primordial germ cells (PGCs), spermatogonial stem cells and sper-

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Table 1
Specification of primers used for gene expression analyses.

Amplicon length	Sequence	Accession No	Name
154bp	F-5'-AATGAGGCAGAGAACACGGACAAC-3' R-5'-GGGACTGGGCTTCACACATTTC-3'	NM.001110178.1	POU5F1
195bp	F-5'-CTCCAGCAGCAGACCTCTCCTTG-3' R-5'-CCTTCCTGTGCCACTCTCACCTT-3'	NM.001146142.1	NANOG
166bp	F-5'-GATGAGTTGCGGGAGATGAG-3' R-5'-TTGGAGAATAGATGGTGGCGT-3'	NM.001012930.1	BCL6B
169bp	F-5'-AGGGATGGACATGGGCAATACAAC-3' R-5'-GCCATTCTGACTGCGGTGGATG-3'	D13225.1	c-KIT
441bp	F-5'-TGAAAAACAACAATGGAAGAAGA-3' R-5'-CTAGACAATCCCTGAGTCTCGTTT-3'	XM.416179.3	STRA-8
274 bp	F-5'-CAGGCGTGGATGGCTAACTC-3' R-5'-CAGAACTCTCCCTCTACCAAATC-3'	AB004836.1	CVH
262bp	F-5'-ATCCTCTTCCACCCGCAACAGTCAG-3' R-5'-TGTCTCTCTTTCATCGGCACCTC-3'	XM.417898.2	PLZF
304bp	F-5'-ATACAACTATCAGGCTCCACCACA-3' R-5'-TGCTCTTCTTTTCTGAAGTGATG-3'	NM.204218.1	DAZL
154bp	F-5'-TGCTTCTCTGTTACCTCTCTG-3' R-5'-ATGACGGCTTGCCTGACC-3'	EF467327.1	SPRY-1
265bp	F-5'-CCTTCATCGATCTGAACACATGG-3' R-5'-GGAGCTGAGATGATAACACGCTTA-3'	NM.204305.1	GAPDH

matogonia (Nayernia et al., 2006; Lue et al., 2007; Heo et al., 2011). Moreover, immunomodulatory effects of mesenchymal stem cells and their great potential to differentiate into many types of cells including osteocytes, cardiomyocytes, neurons and epithelial cells (Benayahu et al., 1989; Makino et al., 1999; Woodbury et al., 2000; Paunescu et al., 2007) make them good candidates for generation of germ cell lineages such as spermatogonial stem cells.

Retinoic acid has been mostly used for differentiating mesenchymal stem cells towards SSCs. The action of this small molecule to differentiate stem cells is believed to be related to induction of *Stra8* gene (Li et al., 2007; Zhou et al., 2008). On the other hand, molecular mechanisms for regulation of self-renewal and maintenance of SSCs are governed by GDNF and associated factors like Bcl6, Lhx1 and Spry1 (Oatley et al., 2006; Hofmann 2008; Costantini 2010). In this study, we compared the effects of GDNF and RA as inducers to test the possibility for switching the chicken MSCs towards SSC-like cells. Treated cells were characterized by expression analysis of some germ cell molecular markers such as *STRA8*, *CVH*, *PLZF*, *DAZL* and factors required for self-renewal and maintenance of SSCs such as *POU5F1*, *NANOG*, *c-KIT* and *BCL6b* (Phillips et al., 2010) at RNA level and germ cell surface markers, TRA-1-60 and TRA-1-81 at protein level.

2. Materials and methods

2.1. Isolation and expansion of cMSCs

cMSCs were isolated from the bone marrow of a 1–10 day old Hy-line chicken. Femur and tibia were soaked in phosphate buffered saline (PBS) supplemented with 2× penicillin/streptomycin. The pieces of broken bones were put on a 70 µm cell strainer and then washed thoroughly by PBS containing the antibiotics remove cell debris. Cells passing through the strainer were washed with PBS twice and cultured in Low glucose-DMEM containing 12.5% FBS (Gibco), 2 mM L-glutamine (Sigma) and 200 µ/ml penicillin/streptomycin, incubated at 37 °C and 5% CO₂. During the first 24 h, the medium was changed every 8 h to get more purified cMSCs and 4 ng/ml basic fibroblast growth factor (bFGF) (Chemicon) was added after 24 h (expansion medium). When the colonies of MSCs were formed, they were first passaged 1:1 using 0.25% trypsin. After that, when cells reached confluency, they were passaged at a density of 1 × 10⁴ cells per cm².

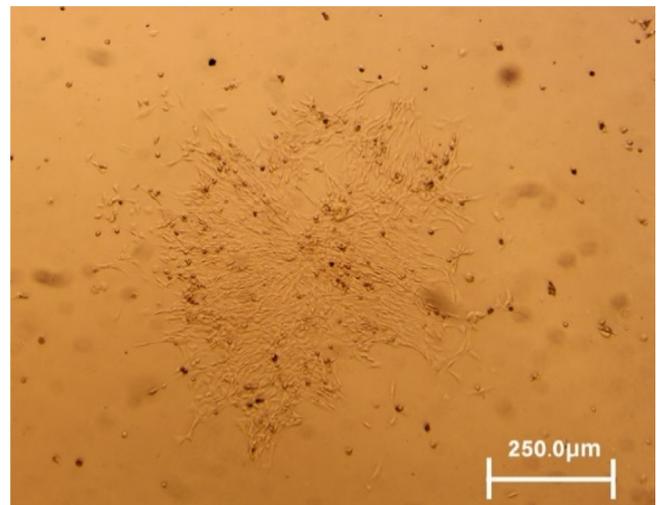


Fig. 1. Primary culture of MSCs isolated from chicken bone marrow. The spindle like morphology and colonial aggregation of cells can be observed.

2.2. Characterization of cMSCs

2.2.1. Differentiation assays

2.2.1.1. Adipogenic differentiation. In order to verify the adipogenic differentiation potency of cMSCs, cells were cultured (~2 × 10⁴ cells/cm²) using expansion medium in 6-well plates. After reaching 80–90% confluency, medium was exchanged with adipogenic induction medium (DMEM supplemented with 10% FBS, 100 µ/ml penicillin/streptomycin, 1 µM dexamethasone, 10 mM β-glycerol phosphate and 100 µM indomethacin) for 21 days. The medium was replaced every 3–4 days and the adipogenesis potential was assessed by Oil Red O staining. To do so, cells were fixed with 4% paraformaldehyde (PFA) and incubated at room temperature for at least 30 min. Fixed cells were washed with distilled water and incubated with 60% isopropanol for 5 min at room temperature. Finally, cells were stained with 0.3% Oil Red O staining solution (Sigma–Aldrich, Germany) and incubated at room temperature for 15 min. In order to visualize the nuclei, cells were stained with hematoxylin.

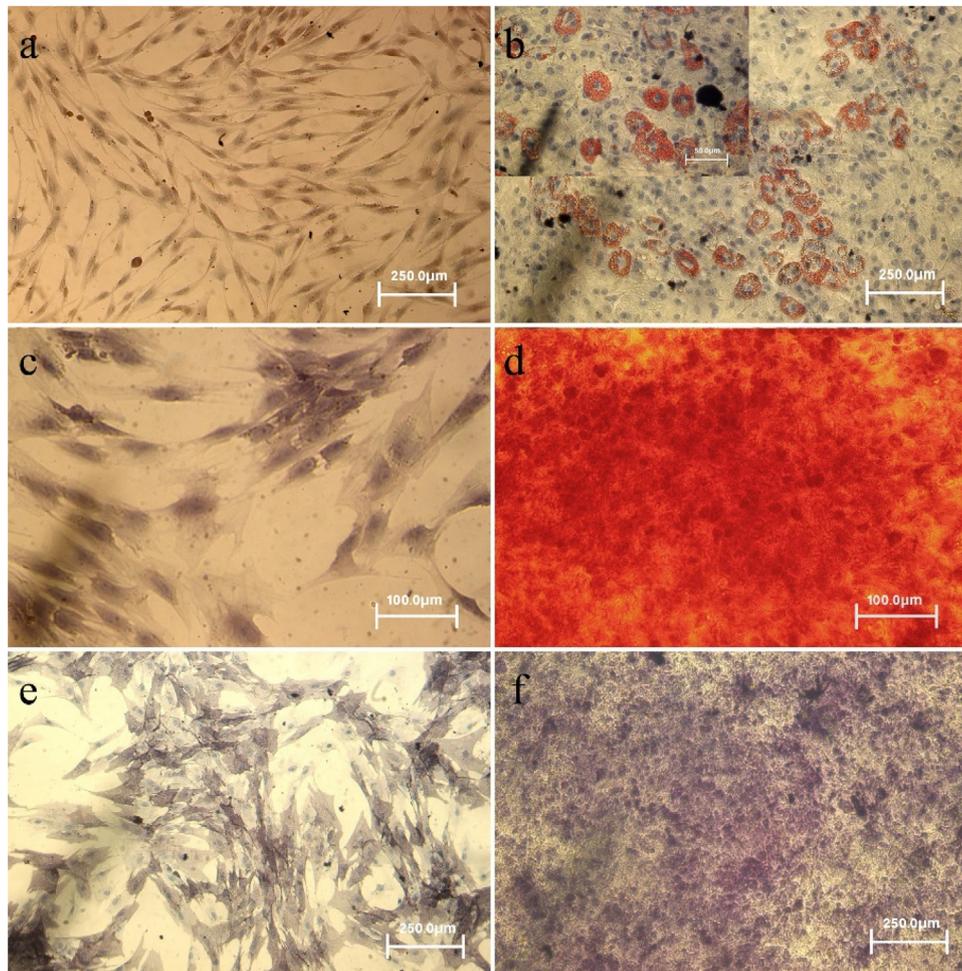


Fig. 2. Differentiation of chicken MSCs to adipogenic and osteogenic lineages *in vitro*. (a and b), adipogenic differentiation. For assessment of adipogenesis, chicken MSCs were cultured in adipogenic medium, followed by staining with Oil Red O and HE (b), untreated chicken MSCs were used as a control (a). (c–f) osteogenic differentiation. For evaluation of osteogenesis cells were cultured in osteogenic medium. Osteogenic differentiation is illustrated by calcium deposition (d), and alkaline phosphatase activity (f), in comparison to control cultures without any induction (c and e) respectively.

2.3. Osteogenic differentiation

Confluent cells were cultured in the osteogenic induction medium (DMEM supplemented with 10% FBS, 100 μ /ml penicillin/streptomycin, 0.1 μ M dexamethasone, 10 mM β -glycerol phosphate and 0.2 mM ascorbic acid 2-phosphate) for 14 days with regular replacement of media every 3–4 days. Osteogenic differentiation was then confirmed by calcium deposit assay and alkaline phosphatase activity assay.

2.3.1. Calcium deposit assay

After carefully washing the cells with PBS, cellular monolayer was covered with 4% PFA and incubated for 30 min. Then washed with distilled water and incubated with Alizarin Red S for 45 min at room temperature in the dark. Finally, cells were washed 4–5 times with distilled water and PBS was added to make them ready for analysis.

2.3.2. Alkaline phosphatase assay

After a primary wash with PBS, cells were covered with 4% PFA for 1 min. Fixed cells were washed with washing buffer (0.05% Tween 20 in PBS) and BCIP/NPT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) (Roche) substrate solution was added to cover the cellular monolayer. Cells were incubated at room

temperature in the dark for 5–15 min and staining process was checked regularly every 2–3 min. Cells were carefully washed with water carefully to remove BCIP/NPT substrate solution and then PBS was added to prepare cells for analysis.

2.3.3. Flow cytometry analysis

SSEA-3, SSEA-4 and GPR125 monoclonal antibodies conjugated with fluorescein isothiocyanate and TRA-1-60 and TRA-1-81 antibodies were used to label cMSCs to analyze stem cell surface markers via FACSCalibur Flow Cytometer (BD) using Flowing software 2.

2.4. Differentiation of chicken MSCs towards SSC-like cells

After characterization of cMSCs, they were subjected to differentiation by treatment with GDNF and RA. Differentiation medium containing DMEM/F12 supplemented with 10% FBS, 100 μ /ml penicillin/streptomycin, 2 mM L-glutamine, 4 ng/ml bFGF and 1 \times β -mercaptoethanol was considered as a master medium. This master medium was supplemented with the two inducers RA (10^{-6} M) and GDNF (15 ng/ml). cMSCs at passage 3 were used for treatment and gene expression profiling was performed after 4, 7 and 14 days of treatments.

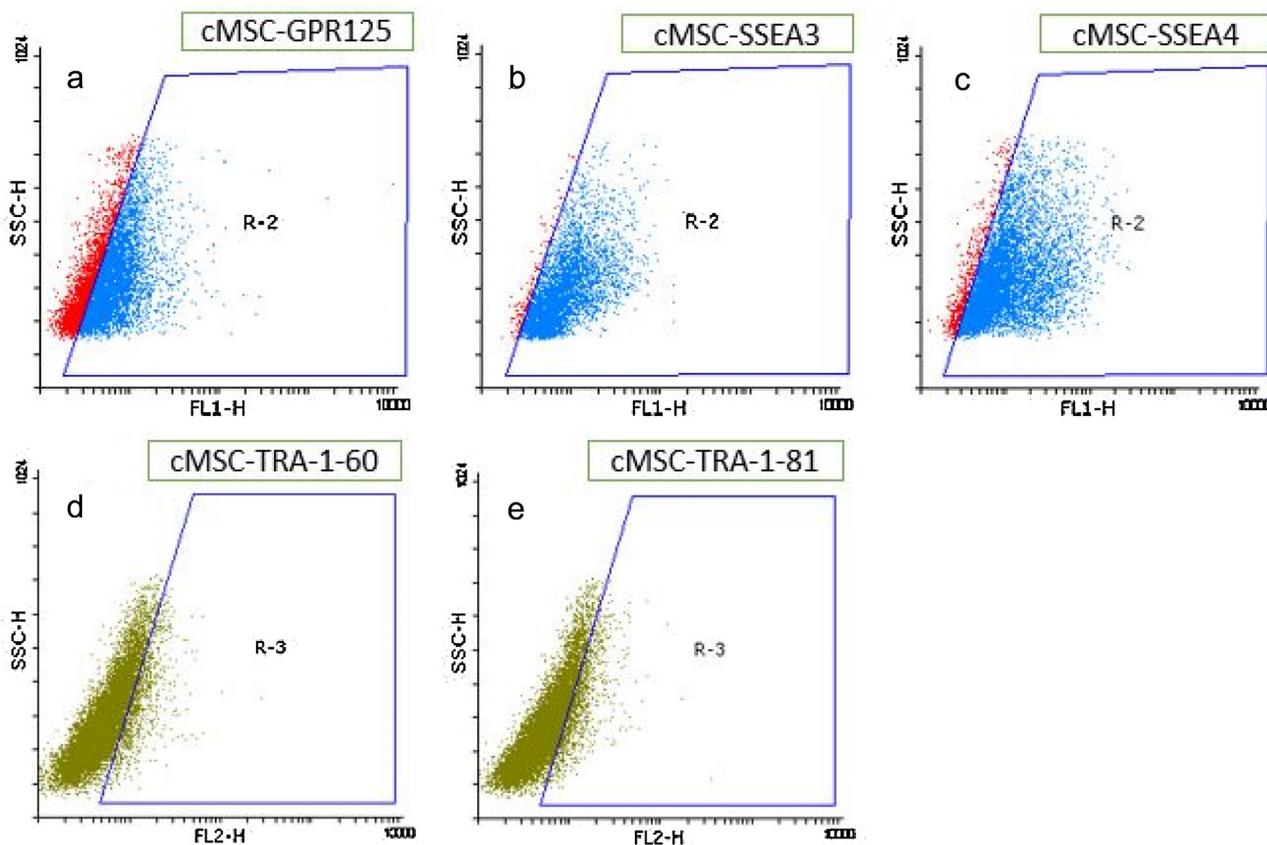


Fig. 3. Flow cytometry analysis of cell surface markers in cMSCs at passage 3. The calculated percentages of the positive cells were: 65% for GPR125 (a), 97% for SSEA3 (b), 93% for SSEA4 (c), 6% for TRA-1-60 (d), and 7% for TRA-1-81(e).

2.4.1. RT-PCR analysis

Total RNA extraction from cMSCs, treated cells and testis tissue, was carried out using Tri-Pure (Roche). Following DNase I treatment, RNA samples were used for cDNA synthesis using MMuLV reverse transcriptase (Fermentas). RT-PCR was then performed for *POU5F1*, *NANOG*, *c-KIT*, *BCL6b*, *STRA8*, *CVH*, *PLZF*, *DAZL*, *GFR α 1*, *GPR125*, *GDNF* and *SPRY1* using their specific primers (Table 1) and negative results were validated by re-PCR.

2.4.2. Flow cytometry analysis

TRA-1-60 and TRA-1-81 antibodies were used to label 7 day-treated cMSCs and analyze these germ cell surface markers by flow cytometry.

3. Results

3.1. Isolation and expansion of cMSCs

Cells, isolated from the marrow of femurs and tibias, grew successfully in culture reaching up to 60–70% confluency after 3 days of initial culturing. The cells had a spindle-like morphology with a tendency to make colonies (Fig. 1).

3.2. Characterization of cMSCs

3.2.1. Differentiation assays

To confirm the identity of cMSCs, their differentiation capacity to adipocytes and osteocytes was examined after culturing in specific adipogenic and osteogenic induction media, respectively. Accumulation of lipids was observed in the intracellular vesicles,

as a sign of adipogenesis (Fig. 2b). Appearance of clear calcium nodules was indicative of osteogenesis (Fig. 2d) and also alkaline phosphatase assay proved the osteogenic differentiation of cMSCs (Fig. 2f), whereas undifferentiated cMSCs did not show the adipogenic or osteogenic characteristics (Fig. 2a, c and e).

3.2.2. Flow cytometry analysis

The results of flow cytometry analysis indicated that cMSCs were highly positive for SSEA-3 and SSEA-4 stem cell markers but weak expression of TRA-1-60 and TRA-1-81 was detected. Moreover, cell surface marker GPR125 was expressed on 65% of the population (Fig. 3).

3.3. Differentiation of cMSCs using defined factors

3.3.1. GDNF treatment

The results of gene expression analysis by RT-PCR illustrated that 4 days after treatment of cMSCs with GDNF, expression of *GDNF* was not detected and these cells could express *c-KIT*, *GFR α 1*, *GPR125* and *SPRY1*. cMSCs treated for 7 days illustrated the expression of some germ cell markers indicating *STRA8*, *CVH* and *PLZF* but not *DAZL*. Expression of some stemness genes like *POU5F1*, *NANOG*, *c-KIT* and *BCL6b* was also observed in treated cells after 7 days, while, *SPRY1* expression was not detected. *STRA8* was the only germ cell marker, which was expressed in GDNF treated cMSCs after 14 days. In all three time intervals after GDNF treatment, *c-KIT*, *GFR α 1* and *GPR125* were expressed (Fig. 4). The expression data are summarized in Table 2.

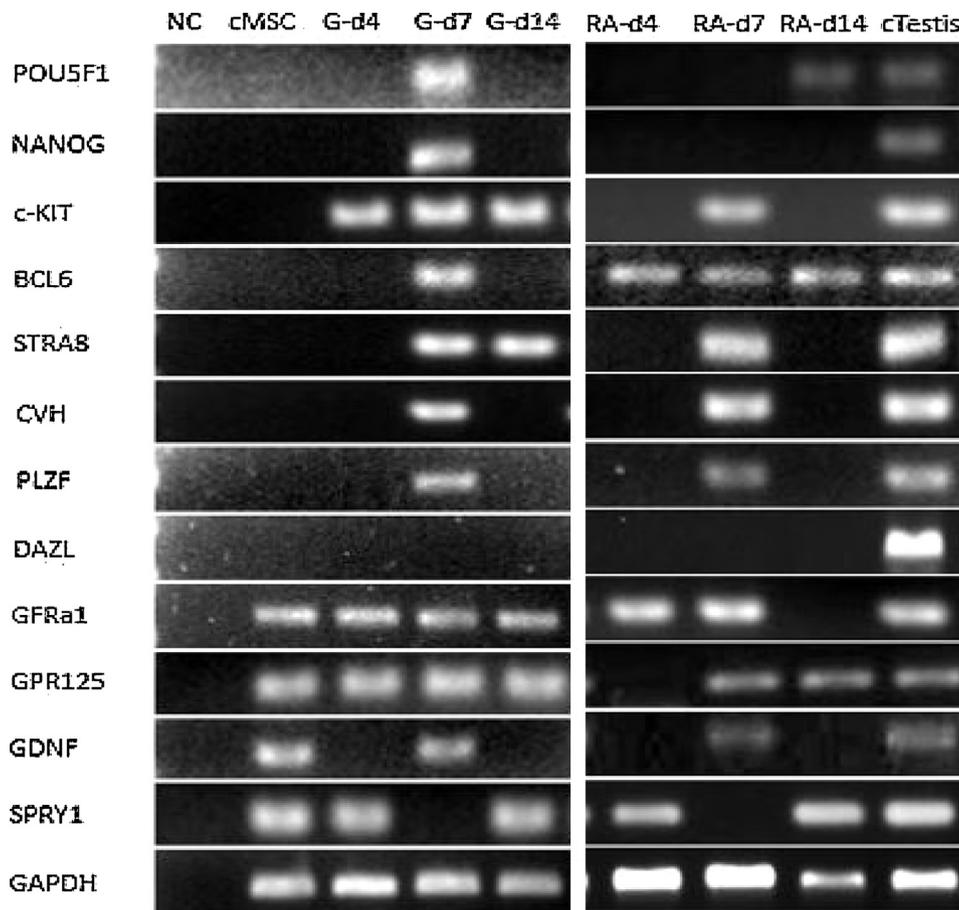


Fig. 4. RT-PCR analysis of cMSCs treated with GDNF and RA during 14 days. NC represents negative control. cMSC represents untreated cMSCs. G-d4, G-d7, and G-d14 represent the GDNF treated cMSCs after 4, 7, and 14 days, respectively. RA-d4, RA-d7, and RA-d14 indicate cMSCs treated with RA for 4, 7, and 14 days, respectively. cTestis represents the chicken testis tissue.

Table 2
Gene expression analysis of cMSCs treated with GDNF and RA.

	POU5F1	NANOG	c-KIT	BCL6	STRA8	CVH	PLZF	DAZL	GFR α 1	GPR125	GDNF	SPRY1
cMSC	–	–	–	–	–	–	–	–	+	++	++	++
cTestis	++	++	++	++	++	++	++	++	++	++	++	++
G-d4	–	–	++	–	–	–	–	–	–	++	–	++
G-d7	+	+	+	++	++	++	++	–	++	++	++	–
G-d14	–	–	++	–	++	–	–	–	++	++	–	++
RA-d4	–	–	–	++	–	–	–	–	++	–	–	++
RA-d7	–	–	++	++	++	++	++	–	++	++	++	–
RA-d14	++	–	–	++	–	–	–	–	–	++	–	++

Abbreviations. cMSC, chicken mesenchymal stem cell. cTestis, chicken testis tissue. G-d4, G-d7, and G-d14 represent the GDNF treated cMSCs for 4, 7, and 14 days, respectively. RA-d4, RA-d7, and RA-d14 represent cMSCs treated with RA for 4, 7, and 14 days, respectively. (–): expression of the gene was not detected, (++) : expression of the gene was detected, (+): expression of the gene was detected after re-PCR.

3.4. RA treatment

After 4 days of RA treatment, cMSCs expressed *BCL6b*, *GFR α 1* and *SPRY1*. Cells treated for 7 days illustrated the expression of *c-KIT*, *BCL6*, *STRA8*, *CVH*, *PLZF*, *GFR α 1*, *GPR125* and *GDNF* genes. 14 days treated cells were positive for the expression of *POU5F1*, *BCL6*, *GPR125* and *SPRY1*. Expression of *BCL6b* and *GPR125* was detected in all RA treated cells by RT-PCR (Fig. 4).

3.5. Flow cytometry analysis of treated cMSCs

Flow cytometry analysis was also performed for verification of the germ cell surface markers. Differentiated cells after 7 days treatment with GDNF and RA, were assessed for expression of germ cell surface markers TRA-1-60 and TRA-1-81 (Muller et al., 2008). 87% of

cMSCs treated with GDNF, showed TRA-1-60 and TRA-1-81 expression after 7 days of treatment (Fig. 5a, b). On the other hand, 7 days of RA treatment resulted in the induction of the two markers 26 and 31% of the cells, respectively (Fig. 5c, d).

4. Discussion

Transgenic production of poultry has been categorized as the most efficient natural bioreactor system and an ideal model for developmental biology (Ivarie 2003; Mozdziak and Petite 2004). Advantages of chicken as a bioreactor among other avians makes it the ultimate goal of many researches (Harvey and Ivarie, 2003). Different strategies have been applied to produce transgenic chickens including direct modification of embryos with DNA or viral vec-

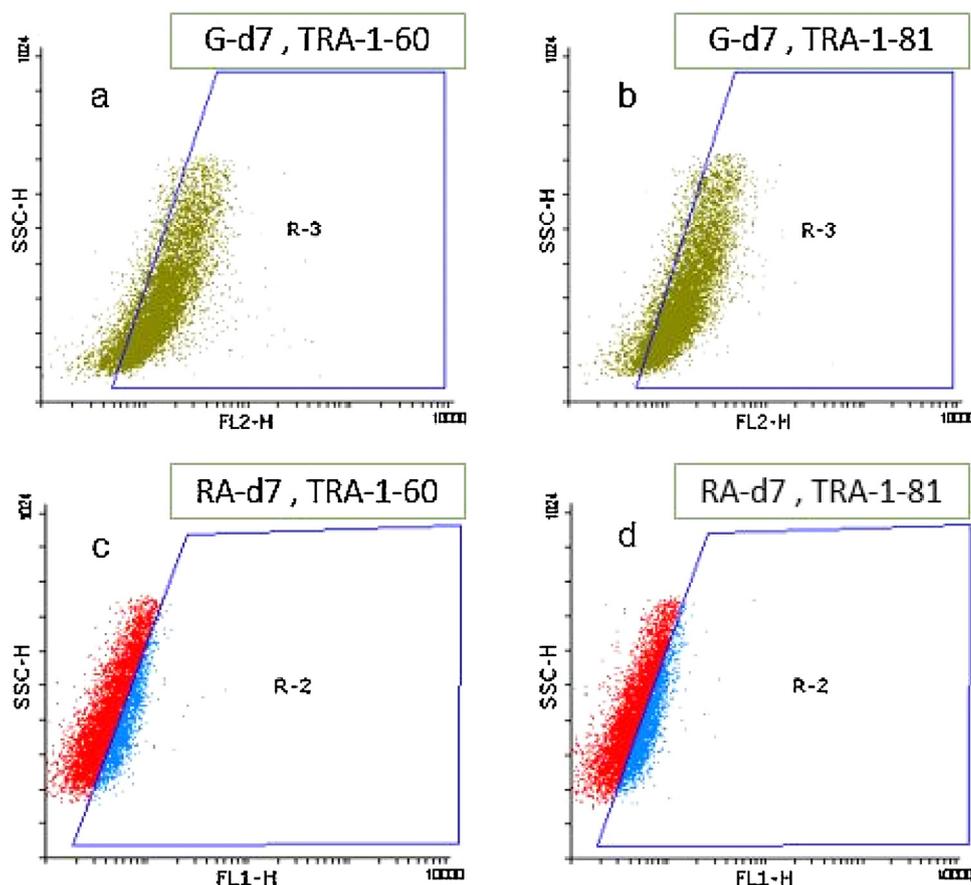


Fig. 5. Flow cytometry analysis of cMSCs treated with GDNF and RA using TRA-1-60 and TRA-1-81 antibodies. (a and b) cMSCs treated with GDNF after 7 days. (c and d) cMSCs treated with RA after 7 days.

tors, cell based protocols, and genetic modifications in embryonic stem cells (ESCs), primordial germ cells or spermatogonial stem cells (van de Lavoie et al., 2006; Li et al. 2008; Motono et al., 2010; Heo et al., 2011; Boozarpour and Momeni-Moghaddam, 2015).

SSCs are pluripotent cells that have recently been considered as a valuable tool for transgenesis (Takehashi et al., 2010) and could be settled in the germline niche due to $\beta 1$ -integrin expression which has a major role in the homing of SSCs (Kanatsu-Shinohara et al., 2008). Since isolation and maintenance of SSCs in culture is a major obstacle, alternative strategies for obtaining SSCs have been introduced like the differentiation of MSCs to SSC-like cells (Nayernia et al., 2006; Heo et al., 2011; Ghasemzadeh-Hasankolaei et al., 2012), however, the molecular mechanism underlying the MSC differentiation toward germ cells remains unclear.

Different inducing factors have been described for differentiation of MSCs to other lineages including RA, which is widely accepted as differentiating factor of MSCs (Rohwedel et al., 1999; Gudas and Wagner 2011). The effects of RA on alteration of gene expression and regulation of stem cell differentiation have been studied (Balmer and Blomhoff 2002; Gudas and Wagner 2011). RA also plays an important role in the proliferation and differentiation of primordial germ cells (Yu et al., 2011) and it has a unique role in differentiation of MSCs in to male germ cells (Nayernia et al., 2006; Heo et al., 2011, Ghasemzadeh-Hasankolaei et al., 2012). On the other hand, RA has a teratogenic capacity since embryonic malformations were observed when an excess amount of retinoids was used (Ghasemzadeh-Hasankolaei et al., 2012). Non-teratogenic inducing factors also remained the quest for scientists to differentiate cells as they do in their natural microenvironment. GDNF was the first identified in conditioned media of glioma cell

line cultures (Lin et al., 1993). GDNF has been described as another differentiation factor of stem cells (Zurn et al., 1996) and as a regulator of SSCs self-renewal (He et al., 2009). It has been termed as a key factor in the SSCs fate determination (Meng et al., 2000).

In this study, chicken mesenchymal stem cells were characterized by their ability to form colonies (Fig. 1) and also their differentiation potential to adipogenic and osteogenic lineages (Fig. 2). These cells highly expressed stem cell markers SSEA3 and SSEA4. Furthermore, expression of GPR125 cell surface marker was detected in 65% cMSCs (Fig. 3). GPR125 has been reported as a SSCs marker in mouse and human (Seandel et al., 2007; Conrad et al., 2008). Similarly, we showed that chicken spermatogonial stem cell colonies could expressed this cell surface marker (Sisakhtnezhad et al., 2015). However, based on flow cytometry analysis of cMSCs, we do not recommend GPR125 as a reliable specific marker for chicken spermatogonial stem cells.

We used RA and GDNF to examine their roles in cMSCs differentiation potential toward SSC-like cells. In both treatments, the properties of treated cMSCs had more similarities to SSCs after 7 days. These cells could express some germ cell markers such as *STRA8*, *CVH* and *PLZF* and they were also positive for *BCL6b* and *c-KIT* which have a role in maintenance of SSCs. Moreover, after 7 days of cMSCs treatment with GDNF, low level expression of *POU5F1* and *NANOG* genes was detected. Therefore, cMSCs treated with GDNF had more similarity to SSCs than RA treated cells. These results were also confirmed by germ cell surface markers TRA-1-60 and TRA-1-81 (Fig. 5). Nevertheless, we could not detect expression of *DAZL* in any treatments which is probably because it is expressed at the late stage of spermatogenesis cycle.

In conclusion, we showed for the first time that GDNF has a potential to differentiate chicken MSCs into SSC-like cells in vitro and we suggest that GDNF has a greater potency than RA, in generating SSCs from cMSCs, however more experiments are required to confirm this.

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