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Pluripotency properties of embryonic stem cells isolated from stage X blastoderm of Mazandaran native chicken

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Abstract

The aim of this study was to isolate Embryonic Stem Cells (ESCs) from native chicken and to characterize their pluripotency properties through the cellular and molecular markers. Samples obtained from fertilized eggs from Mazandaran native hens. Cells were isolated from area of pellucida from stage X native hens' blastoderm. Then the cells were cultured on inactivated mouse SNL feeder cells in the presence of *LIF*, *IGF-1*, *bFGF*, *CNTF*, *OSM*, *SCF*, *Il-6*, and *Il-11* growth factors. The native chickens' ESCs colonies were picked up and subsequently passaged. To characterize the cells, they were analyzed for their alkaline phosphatase activity, and also for the expression of *SSEA-4*, and *TRA-1-60* as embryonic-specific markers at the protein level. Furthermore, the expression of pluripotency (*cPouV*, *Sox2*, and *Nanog*) and cell lineage specific (*Cvh*, *Brachyury*, and *Gata6*) gene markers was evaluated at the level of mRNA using quantitative RT-PCR. Isolated cells were passaged repeatedly and successfully up to ten passages. The stemness of embryonic cells has been approved by the activity of the alkaline phosphatase, presence of the *SSEA-4*, and *TRA-1-60* protein, and expression of the molecular marker (*cPouV*, *Nanog*, and *Sox-2*) genes. The spontaneous differentiation of chicken ESCs confirmed the pluripotecy of the cells in differentiation into specialized cell lineages. Our observation showed that ESCs can be isolated successfully from stage X blastoderm of Mazandaran native chickens and these cells maintain their stemness properties during multi-passages *in vitro*.

Key words: Chicken, egg, blastoderm, embryonic stem cell, stemness marker.

Introduction

Stem cells can broadly be divided into two cell type: embryonic stem cells (ESCs) and adult stem cells (1, 2). In comparison to adult stem cells, ESCs are generally more potent and can potentially differentiate into all three-germ layers-derived cell lineages (3). Pluripotent stem cells with embryonic stem (ES)-like cells properties and adult stem cells have been successfully isolated and characterized extensively in a variety of research studies (4-8). In the last decade, establishment and maintenance of the pluripotent ESCs have reached their greatest notoriety in stem cell biology and regenerative medicine (9, 10). As ESCs became more widely used in experimental studies, application of these cells also showed promising results in clinical trials (11-13).

ESCs are a powerful source for generating potentially unlimited numbers of specialized cells (14). These cells were first derived in 1981 in the mouse (15) and subsequently from human blastocysts and a number of other species such as mink (16-18), rhesus monkey (19), pig (20), and hamster (21). Likewise, chicken (Gallus gallus domesticus) embryonic stem cells (ESCs) are derived from cells obtained from stage X embryos (blastoderm stage); and, as reviewed by Intarapat and Stern (2014), chicken ESCs have the ability to contribute to all somatic lineages in chimaeras, but not to the germ line (22). Not only the chicken is considered as premier model in developmental biology (23), but also it can be used as bioreactor for the production of exogenous proteins in a egg white (24). Because of its simplicity of isolation, unique properties, and ease of processing compared to other source of stem cells (25, 26), chicken ESCs are a valuable source of cells that can be used to investigate the molecular mechanism of differentiation and pluripotency (27-29).

Conservation of animal genetic resources is a topic of discussion since the 1950s (30). The genetic resource base of the indigenous animals could form the basis for genetic improvement and diversification to produce breeds adapted to local conditions (31). Among the multiple guidelines and strategies which have been proposed for maintaining the genetic resources of both domestic and wild animals (32-34), isolation and culture of ESCs as an efficient approach appropriately applied in various species (35, 36). As per literature survey, this widely applied approach has not been evaluated in native chicken.

The objective of current study, therefore, was to isolate and characterize the ESCs from native fowls for saving population of native breed in rural areas. To accomplish this, ESCs were isolated from stage X blastoderm of native chickens and characterized by the

presence of typical ESC markers such as activity of the alkaline phosphatase and presence of specific proteins like SSEA-4 and TRA-1-60. To further characterized the derived ESCs, expression of pluripotency-related (*cPouV*, *Sox2*, and *Nanog*) and differentiation-related (*Cvh*, *Brachyury*, and *Gata6*) genes has been evaluated in the cells quantitatively. Finally the potency of the established ESCs in generation of specialized cell lineages was tested in the mRNA level.

Materials and methods

All products were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) and Gibco (Grand Island, NY, USA) unless stated otherwise.

Isolation and culture of cells

Fertilized eggs from Red Naked Neck hens, a native chicken of Mazandaran native chicken were transported to the laboratory and cleaned with 70% ethanol before processing to reduce the risk of contamination. The area of pellucida (Fig. 1) was isolated from stage X blastoderm, washed twice with culture medium, and dispersed into a single cell suspension by a 200 µl pipette. Then the single cells were seeded and grown on feeder layers of mitotically inactivated SNL 76/7 cells treated with mitomycin-C (Invitrogen Co. USA) in 6-well gelatinized culture plates (Orange Science, Belgium). ESCs were cultured on a feeder layer in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20 % fetal bovine serum (FBS), 1% non-essential amino acid, streptomycin (100 µg/mL), penicillin (100 U/mL), 10 ng/ml gentamycin, 0.1 mmol/l b-mercaptoethanol, 20 ng/ml LIF, 20 ng/ml IGF-1,10 ng/ml bFGF, 20 ng/ml CNTF, 20 ng/ml OSM, 10 ng/ml SCF,20 ng/ml Il-6 and 10 ng/ml Il-11 growth factors (all from PeproTech). SNL76/7 cells were cultured and passaged in DMEM supplemented with 20 % FBS. Noninfected MDCC-MSB1 cells also were cultured in media containing high glucose DMEM and 10 % FBS and processed for control purposes.

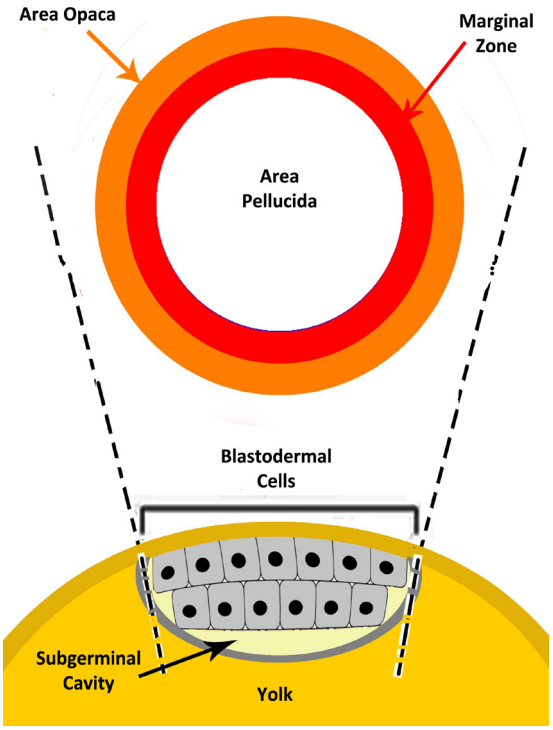

Figure 1. Schematic illustration of stage X chicken embryo. The upper figure shows the ventral view, and the lower figure shows the cross section. Two distinct regions of blastoderm can be identified, the area pellucida and the area opaca, consisting of the darker cells at the margin of the blastoderm and yolk. Between the area pellucida and the yolk is a space called the subgerminal cavity (60).

Pluripotency characterization and maintenance

Alkaline phosphatase staining of ESCs

ESCs maintained on SNL 76/7 cells for 10 passages were isolated and evaluated for the activity of alkaline phosphatase. For this, after washing with phosphate buffer saline (PBS), cultured cells were fixed with cold 4% paraformaldehyde at 4°C for 20 minutes. Alkaline phosphatase staining solution containing 100 mM NaCl, 100 mM Tris-HCl pH 9.5, 5 mM MgCl2, 1 mg/ml NBT, 0.1 mg/ml BCIP was added after washing and incubating at 37°C. The reaction was incubated for 5-30 minutes at 37°C, stopped by addition of 10 mM EDTA, and the wells were washed with PBS. Colored colonies were investigated using an inverted microscope (Moticam 2300, 3.0 M pixel USB 2.0).

Immunocytochemistry characterization of ESCs

ESCs were characterized by immunocytochemistry using antibodies against anti-SSEA-4 and TRA-1-60 (Stemgent, USA) as described previously (3). Briefly, after 10 passages ESCs were fixed in 4% of paraformaldehyde for 20 min followed by permeablization with 0.1% TX-100 in TBS (TBST), the cells were blocked for 30 min at room temperature with 5% BSA in TBST. Then, cells were incubated with primary antibodies (1:100 dilution) diluted in 1% BSA in TBST for 1h at RT. Anti rabbit IgG conjugated with R Phycoerythrin (PE) diluted in 1% BSA in TBST (1:400 dilution) were used as secondary antibodies for 30 min at RT. After washing with PBS, cells were incubated with DAPI (4′, 6-diamidino-2-phenylindole; 1:1000, 30 second, sigma, Germany) for nuclear staining. Finally, images were trapped by a fluorescence microscope, Nikon TE200 inverted fluorescence microscope.

RNA isolation, synthesis of cDNA and reverse transcriptase-polymerase chain reaction detection of marker genes

Total cellular RNA was extracted using Qiazol according to manufacturer's instruction (Qiagen, Germany). For this, samples were lysed in 1 ml Qiazol/g tissue by Tissue Lyser LT. Then, the total RNA was extracted from the lysed cells of each sample. Synthesis of cDNA was carried out with M-MuLV reverse transcriptase (RT) and random hexamer as the primer, according to the manufacturer's instructions (Fermentas). PCR amplification was performed using a standard procedure with Taq DNA Polymerase with denaturation at 94 °C for 15 s, annealing at 55-60 °C for 30 s according to melting temperature of each primer, and extension at 72 °C for 45 s. The number of cycles varied between 30 and 40, depending on the abundance of particular mRNA. The primers and product lengths are listed in Table 1.

Table 1. Details of primers used for real time PCR quantitative analysis.

Gene name	Primer sequences	Annealing temperature (°C)	Product size (bp)
Nanog	F: 5' -CCTACAAGCAGGTGAAGACG-3' R: 5′-GCCTGAAGGTTTCTGTTGGC-3'	59	187
cPou v	F: 5' -GGTATCTCGAGCCATTCACCG-3' R: 5'- CATCTTCCCATAGAGCGTGCC-3'	60	165
Sox2	F: 5'-GCAGAGAAAAGGGAAAAAGGA-3' R: 5'-GTGGAAAGGTGGCATGTAGAC-3'	54	170
Cvh	F: 5'-TGTCATCCCTGTTTTGCTTG-3' R: 5'-ACAAATCCTGCGGAAAATTG-3'	53	158
Brachyury	F: 5'-ACGCCATGTACTCCTTCCTG-3' R: 5'-TGTTGGTGAGCTTGACCTTG-3'	56	198
Gata6	F: 5'-CCGACCACTTGCTATGAAAAA-3' R : 5'-CAGCCCATCTTGACCTGAATA-3'	54	274
Rs17	F : 5'-ACACCCGTCTGGGCAACGACT-3' R : 5'-CCCGCTGGATGCGCTTCATCA-3'	63	129

PCR, polymerase chain reaction; F, forward; R, reverse.

Real-time PCR of marker genes

Real-time PCR reactions in 25µl final volume containing 12.5 µl 2X SYBR Premix Ex Taq (Takara Bio Inc.), 0.4 mM of final concentration for each primer, 2 µl template and distilled water were conducted in Rotor Gene 6000 (Corbett life science). Real-time PCR was performed in a two-step program with the following thermal setting: 3 min at 95 °C for initial denaturation followed by 40 amplification cycles (each 5 s at 95 °C, 20 s at 60 °C with fluorescence detection) and a final step of melting curve analysis. All the samples were analyzed in duplicate, and the average value of the duplicate was used for quantification. The data were normalized to *Rs17* and $2^{-\Delta\Delta Ct}$ methodology was used for relative quantification (37).

Statistical Analysis

All data are expressed as the mean±SD of at least three experiments. Statistical analysis was done using the one-way analysis of variance (ANOVA) to compare the results. Values of $p < 0.05$ were considered to be statistically significant. All statistical analyses were conducted with SPSS software, version 17.0.

Results

Morphology and growth properties ESCs

Our data showed that native hens ES-like cells are maintained as self-renewing pluripotent cells on feeder cells. Inactivated SNL76/7 feeder cells showed in Figure 2.A, and formation of typical circle-shaped colonies were observed after one day culture of the area pellucida on inactivated feeder layers. After one week, ESCs colonies get sufficiently large to cover ~50% of the well and became more visible with distinct boundary (Fig. 2B).

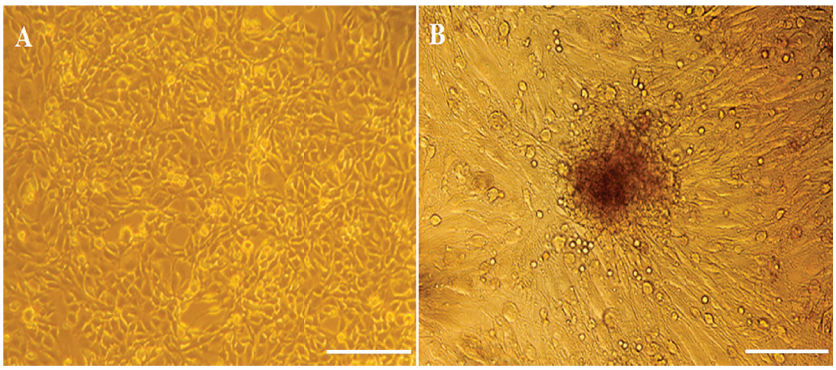

Figure 2. Morphology of native chicken embryonic stem cells is shown. The SNL cells (A) used as feeder cells. The embryonic stem cells made large colonies (B) a day after culture on SNL feeder cells. Scale bar is 65µm.

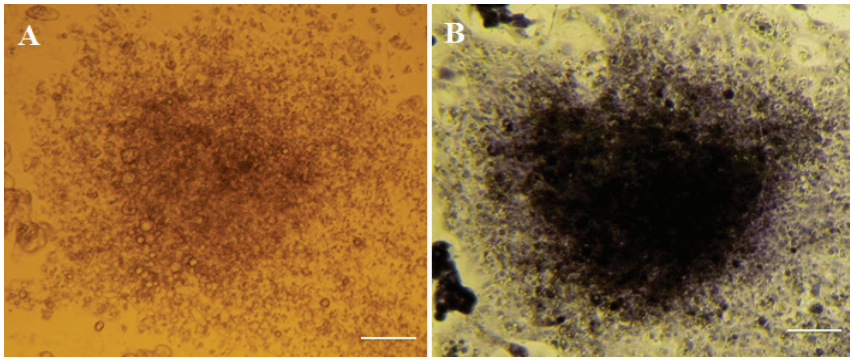

Figure 3. A colony of undifferentiated ESCs (A) was characterized for the alkaline phosphatase activity which is distinguishable by violet dye (B). Scale bar is 65µm.

Pluripotency characterization

Alkaline phosphatase staining of ESCs

After 10 passages, the positive colonies with a compact and round morphology were scored as ESCs colonies. Five colonies were selected on the basis of morphological criteria. Figure 3.A shows a colony before staining for the alkaline phosphatase. For detection of alkaline phosphatase activity, the colonies were fixed and stained with crystal violet. The positive colonies for alkaline phosphatase protein demonstrated violet dye in contrast with SNL76/7 feeder cells (Fig. 3B). The observation of 20 microscopic fields with 10X magnification showed no negative colonies for alkaline phosphatase.

Immunocytochemistry characterization of ESCs

The isolated stem cells were evaluated for expression of stemness-related cell-surface antigens, SSEA-4 and TRA-1-60 (Fig. 4). The results showed presence of SSEA-4and TRA-1-60 (Fig. 4C and F, respectively) on the surface of ESCs whereas the expression of these stem cell markers was not detect in SNL76/7 cells as control (Fig. 4I). The observation of 20 different fields of immunostained cells under fluorescent microscope showed that all morphologically defined ES colonies are positive for both SSEA-4 and TRA-1-60 markers; however the possible presence of single ES cells which did not express these markers cannot be excluded.

Gene expression of marker genes

In order to further confirm the pluripotency of ESCs derived from native chickens, we evaluated the expression abundance of candidate marker genes using real-time PCR. For this, the expression of candidate genes for stemness (*cPouV*, *Sox2*, and *Nanog*) and cell linage-specific (*Cvh*, *Brachyury*, and *Gata6*) were evaluated at the level of mRNA in ESCs while compared with chicken adult tissue and a chicken cell line as control groups. Expression data for stemness (*cPouV*, *Sox2*, and *Nanog*) genes and cell lineage-specific (*Cvh*, *Brachyury*, and *Gata6*) genes in ESCs as compared with MDCC cell line and, chicken adult tissue are presented in Figure 5a and 5b, respectively. Our results showed that the relative transcript abundance of *cPouV*, *Sox2*, and *Nanog* was increased ($P < 0.05$) in ESCs compared to that of control cells. We also observed a decreased (P

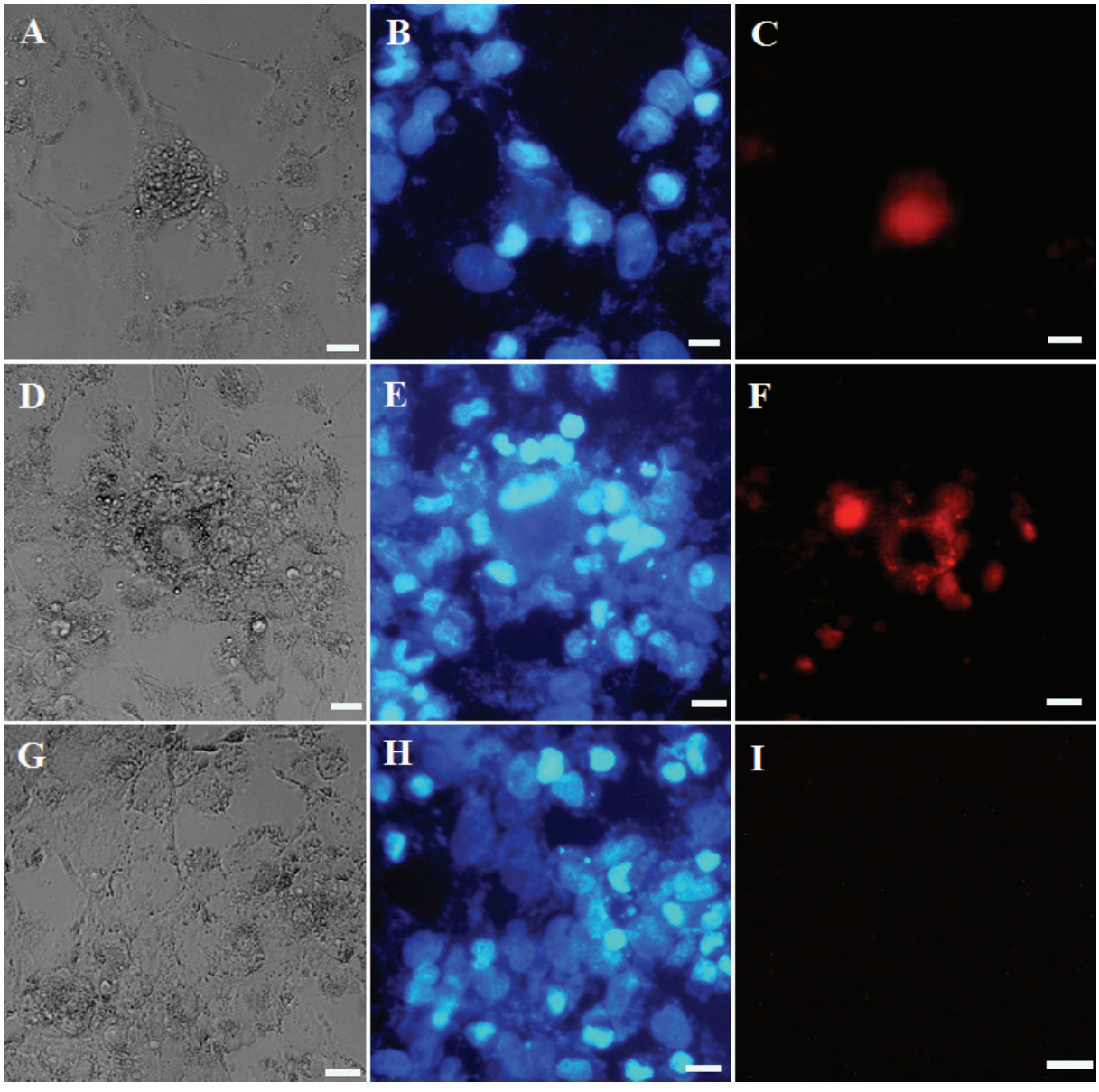

Figure 4. ESCs were expressed SSEA-4 (A, B and C), and TRA-1-60 (D, E, and F) proteins as determined by immunocytochemical analysis. SNL feeder cells as control cells did not express SSEA-4 and TRA-1-60 proteins (G, H, and I). Scale bar is 10µm.

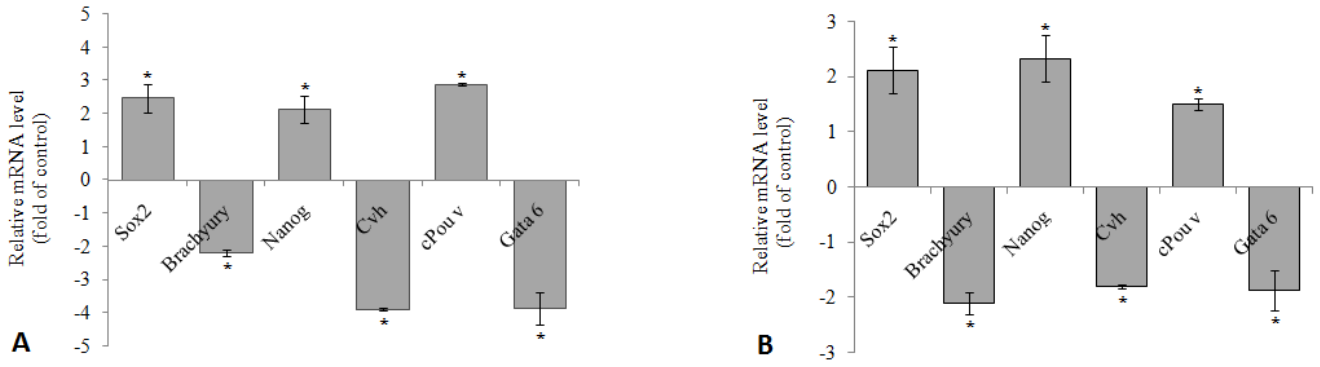

Figure 5. Expression of pluripotency (cPouV, Sox2, and Nanog) and differential (Cvh, Brachyury, and Gata6) gene markers in ESCs compared with MDCC cell line (A) and compared with the tissue of adult chicken (B).

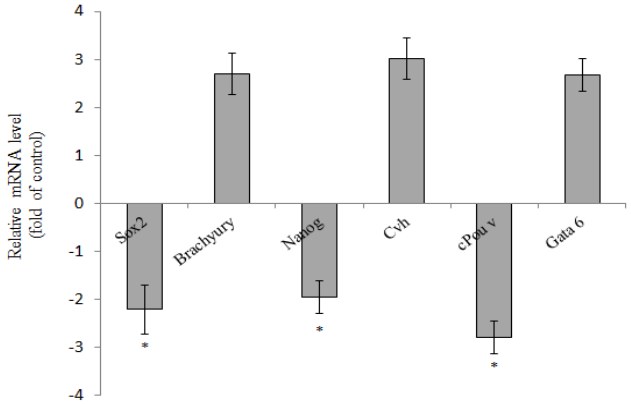

Figure 6. Expression of pluripotency (cPouV, Sox2, and Nanog) and differential (Cvh, Brachyury, and Gata6) gene markers in ESCs compared with MDCC cell line after growing the cells for spontaneous differentiation.

< 0.05) expression of *Cvh*, *Brachyury*, and *Gata6* transcripts in ESCs in compression with the control cells.

To evaluate the pluripotency of established ESCs, the cells were cultured without the growth factors and also LIF to spontaneously differentiate for three weeks. Then, the expression of Gata6 (expressed in adult mesoderm and endoderm-derived organs), Brachyury (expressed early in mesendoderm then limited to mesoderm) and *Cvh* (expressed in germ cells) were sought. As shown in figure 6, the expression of these markers have been increased in differentiated cells implying the potency of these cells in generation of specialized tissues.

Discussion

The native chicken breeds are becoming endangered or even extinct mainly due to their poor commercial performances (38). Accordingly, to save native chickens from extinction, several strategies and approaches has been proposed among which isolation of pluripotent stem cells might have a significant contribution to the establishment of embryonic stem cell line (39-42), conservation and utilization of genetic resources (43). In parallel to their important application in conservation of genetic resources, ESCs are major inducers used in transgenesis (44-46), and can be cultured and used for production of transgenic birds as biological bioreactors (24, 47).

Mazandaran is an important pole of agriculture and animal husbandry of Iran (48-50) and approximately have 4,000,000 native chickens (51). Native chicken breeding station of Mazandaran was established in 1988 with two main objectives: genetic improvement through selection programs and protection of indigenous Mazandarani birds (52). With the ultimate goal of establishing a stem cell bank for Mazandaran native chicken, in this work we tried to set up an optimum culture conditions for growth and characterization of ESCs in native chickens. Toward this goal, we isolated embryonic stem cells derived from stage X blastoderm of native chicken and characterized their pluripotency features at the cellular and molecular level.

In this study, ESCs were successfully isolated from stage X blastoderm of native chicken with relative simplicity and speed. The isolated cells maintain their morphological and growth characteristics and were capable of unlimited, self-renewing proliferation in vitro under our appropriate conditions. The growing cells were characterized for alkaline phosphatase activity. Alkaline phosphatase is an enzyme long-known to be expressed in ESCs and down-regulated by differentiated cells (53). After one passage under basal medium supplemented with *LIF*, *IGF-1*, *bFGF*, *CNTF*, *OSM*, *SCF*, *Il-6* and *Il-11* growth factors we packed up several typical "ES-like" morphological features colonies. A high endogenous alkaline phosphatase activity of these cells was detected after 10 passages under ESC growth conditions. Various growth factors have been reported to act on proliferation of ESCs as well as differentiation prevention of these cells (54-57). Here for the first time we tried to optimize an efficient culture condition for proliferation of ESCs in native chickens. Supplementation of some of above the mentioned growth factors was found to be necessary in our culture condition for proliferation of ESCs in native chickens (data not shown) which were consistent with some reports (22, 58), but conflicted with others (59, 60).

In order to further characterize the growing cells, we evaluated the presence of the SSEA-4 and TRA-1-60 proteins in ESCs. The putative ESCs colonies were positively characterized for SSEA-4 and TRA-1-60 by Immunocytochemistry. Furthermore, expression of two groups of genes, those required for pluripotency (*cPouV*, *Sox2*, and *Nanog*) and those required only for differentiation (*Cvh*, *Brachyury*, and *Gata6*), were evaluated quantitatively in ESCs and compared with chicken adult tissue and MDCC chicken cell line as control cells. Our data showed that the expression of all

candidate genes for pluripotency was increased while compared with control cells. It has been evidenced that transcription factors *Nanog*, *Sox2*, and *cPouV* are of central importance in both human and animal ESCs, and play a critical role in normal proliferation of these cells (61-63). Chicken *cPouV* gene is also a main member of pluripotency related gene network and critically involved in the self-renewal of undifferentiated embryonic stem cells (61, 64). Among differentiation related genes which were evaluated in this study, *Gata 6* and *Brachyury* genes are endodermal and mesendodermal markers, respectively. Cvh (chicken vasa homologue) is also a gene that expresses in germ cells. For all of these candidate genes, we found a decrease of expression in ESCs as compared with the control cells. On contrary, the expression of these genes were upregulated when the ESCs were spontaneously differentiated indicating that ESCs are pluripotent.

Taking together, the results of this study demonstrate that ESCs keeps the stemness and pluripotency features after long-term in vitro culture, and 10 passages. In addition, absence of differentiation genes expression in cultured ESCs indicated that the cells have not start any differentiation in vitro. Chicken ESCs were also cultured in vitro for more than 10 passages and maintained in -196°C for using in other study. In conclusion, our results showed that ESCs be able to long-term culture in vitro with maintain all properties and advantages of embryonic and pluripotency.

Acknowledgements

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