

# An Efficient in Vitro Culture System to Amplify Spermatogonia Stem Cell Markers



Zeinab Narimanpour<sup>1</sup> , Maryam Nazm Bojnordi<sup>1,2\*</sup> , Hatf Ghasemi Hamidabadi<sup>1,2</sup> 

1. Department of Anatomy & Cell Biology, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran.  
2. Immunogenic Research Center, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran.



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## ABSTRACT

**Background:** Proliferation of Spermatogonial Stem Cells (SSCs) can be a treatment for infertile men. Here, we design an efficient method based on culturing in the presence of Sertoli cells to improve the expression level of some specific spermatogonia stem cell genes during two weeks post culture.

**Materials and Methods:** Cells were derived from neonatal (2-6 days old) mice testes and were cultured in DMEM medium with FBS. The colonization of cultured SSCs in days 4, 7, and 14 of culture was counted via phase-contrast microscope and Image J software. Methyl Thiazolyl Tetrazolium (MTT) test was performed to evaluate the viability of cultured SSCs in days 3, 7, and 14 of culture. The expression level and the alteration pattern of specific spermatogonial markers, i.e. *Stra8*, *DAZL*, and *Piwill2* was examined via real-time Polymerase Chain Reaction (PCR) during two weeks post culture.

**Results:** The number and the diameters of colonies showed a significant increase in cultured cells. MTT results proved the higher viability of testicular cells during the culture period. The results of ALP staining detected a positive reaction in spermatogonia colonies. Real-time PCR data showed that culturing SSCs in the presence of interstitial cells of the testis, amplified the level and alteration pattern of specific spermatogonia stem cells genes beneficial in the enrichment of SSCs propagation.

**Conclusion:** Providing a similar culture environment to testicular niche increases viability, forms SSCs colonies, and regulates the level and alteration pattern of spermatogonia stem cell genes.

## Introduction

S

permatogonial Stem Cells (SSCs) transport genetic information from one generation to the next one via spermatogenesis and sperm production [1]. They are located in the basal lamina of seminiferous tubules of the testis, surrounded by cellular network

consisted of interstitial cells that act as an SSCs feeder layer and regulates nutrition and proliferation of SSCs [2, 3].

Some elements, especially glial cell-derived neurotrophic factor (GDNF) mediates the survival and proliferation of SSCs [4, 5]. It seems that GDNF is an essential factor for in vitro propagation and colony formation of SSCs [6, 7]. The niche of testis includes extracellular ma-

### \* Corresponding Author:

Maryam Nazm Bojnordi, PhD.

Address: Department of Anatomy & Cell Biology, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran.

Phone: +98 (912) 8102359

E-mail: bojnordi@modares.ac.ir

trix, consisting of Sertoli cells controlling SSCs proliferation using the secretion of GDNF [8-10]. Based on the regulatory effects of Sertoli cells on SSCs, we designed a culture system to improve the stemness profile and proliferation activity of SSCs. To this aim, *Piwill2*, *Stra8*, *DAZL* genes were examined after two weeks of culture.

Previous results support that the enrichment of spermatogonia stem cells in vitro, before their transplantation into the testis of infertile men, may have more effects on the treatment of male infertility.

Here, we design an efficient culture system that is similar to testicular niche to improve the viability and colony formation (as a marker of SSCs proliferation). The number and diameters of colonies increased in the experiment group compared to the control group. This results from the stimulatory effect of Sertoli cells via their interactions with SSCs in vitro culture. Our system amplified the specific level and alteration pattern of spermatogonia stem cell genes which are applicable in the enrichment of SSCs propagation.

## Materials and Methods

### Isolation of spermatogonial cells

Bilateral testes of 2-6 days old mice were collected for testicular cells. After scarifying the animals, their testes were collected and then were placed on ice. At first, the testes were cut into pieces and then digested with a solution containing Dulbecco's Modified Eagle's Medium (DMEM), collagenase-dispase, and trypsin) 0.5 mg/mL). After centrifugation, the cell aggregates were sheared gently via pipetting for 5 min.

In this study, two groups were designed: the experiment group with testicular cells consisting of SSCs cultured with Sertoli cells and the control group with just SSCs. Differential plating method was used for the separation of SSCs from the interstitial cells. Interstitial cells were attached to the bottom of the dish following 12 hours of incubation, while SSCs would remain in the suspension.

### Cell culture

The cells are cultured together in DMEM containing 15% Fetal Bovine Serum (FBS), 1 mM L-glutamine, nonessential amino acids) 0.1 mM (at 37°C, 5% CO<sub>2</sub>, in a humidified atmosphere.

In the control group, the isolated spermatogonial cells were cultured alone. Testicular cells were attached af-

ter 48 h and the medium was refreshed every two days. Some cells such as Sertoli cells attach to the bottom of the dish and form a monolayer and some spermatogonial small colonies are seen on the top of the Sertoli feeder layer. The colonies were trypsinized and passaged every 4 days. Spermatogonia and interstitial cells were cultured for 2 weeks. The cell counting was done with a hemocytometer before culture [11].

### Number and diameters of SSCs colonies

Colony formation was monitored daily via a phase-contrast microscope. Spermatogonial colonies were assayed in the 4<sup>th</sup>, 7<sup>th</sup>, and 14<sup>th</sup> days [12].

### MTT test

Viability of cultured SSCs was measured using 3-(4,5-dimethylthiazoyl-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. A total of 2×10<sup>4</sup> cells in day 4<sup>th</sup>, 7<sup>th</sup>, and 14<sup>th</sup> of culture were transferred to 24-well plates. Then dimethyl sulfoxide (DMSO) 50 µL, was replaced. Absorbance was measured in a Cytofluor 4000 plate reader [13].

### Alkaline phosphates assay

After washing SSCs colonies with PBS, the cells were exposed to Fast Red Violet and a-naphthol phosphate and tested under a microscope.

### Real-Time PCR

After two weeks of post culture, SSCs were examined for *DAZL*, *Stra8*, and *Piwill2* genes. RNA extraction was done using RNX-Plus Kit (Sinagene Co.). The extracted RNA was converted to cDNA and PCR was carried out via adding cDNA for 35 cycles SYBR Green and Master Mix. The PCR program included an initial melting cycle, at 94°C for 4 min, and was succeeded by 40 cycles as follows: denaturation at 95°C for 20 s, annealing at 58°C for 30 s, and elongation at 72°C for 30 s. The quality of the reactions was confirmed by melting curve analyses. The target genes were normalized to the reference gene. The comparative CT method (2<sup>ΔΔCT</sup>) was used to determine the relative quantification of target genes and normalized to a house-keeping gene (β-actin). Primer sequences include:

*Stra8*: 5'-ACGACGCGTCGCTATTCCCTCTCA-CATCTTC-3'

5'-AGCGAGCTCGATGCACCTTCGACACTTG-3'

*DAZL*: 5-GGAGCTATGTTGTACCTCC-3

5-CCATGTAAGTAGATAAGCCAG-3

*Piwill2*: 5'-GCACAGTCCACGTGGTGGAAA-3'

5'-TCCATAGTCAGGACCGGAGGG-3'

$\beta$ -Actin: 5'-CTTCTTGGGTATGGAATCCTG-3'

5'-GTGTTGGCATAGAGGTCTTTAC-3'

### Statistical analysis

One-way Analysis of Variance (ANOVA) was used and Data were presented as Mean $\pm$ SD and P<0.05 was considered significant.

## Results

### Colonization ability of SSCs

The morphological evaluation showed that interstitial cells formed a monolayer that spermatogonial colonies were grown on top of this layer. The colony numbers and size in the co-culture group were increased during the culture period (Figure 1A) compared to the control group (Figure 1B). The number and size of SSC colonies in the experiment group showed a significant increase in comparison to the control group (Figure 2).

### The survival rate of cultured testicular cells

The viability of cultured cells was, 94.70 $\pm$ 0.25%. The percentages of viability in the control group were higher than in the control group (P<0.05) (Figure 4).

### Alkaline phosphates reactivity

The results of ALP staining revealed a higher reaction in SSC colonies in the experiment group compared with the control group (Figure 3).

### Quantitative real-time PCR

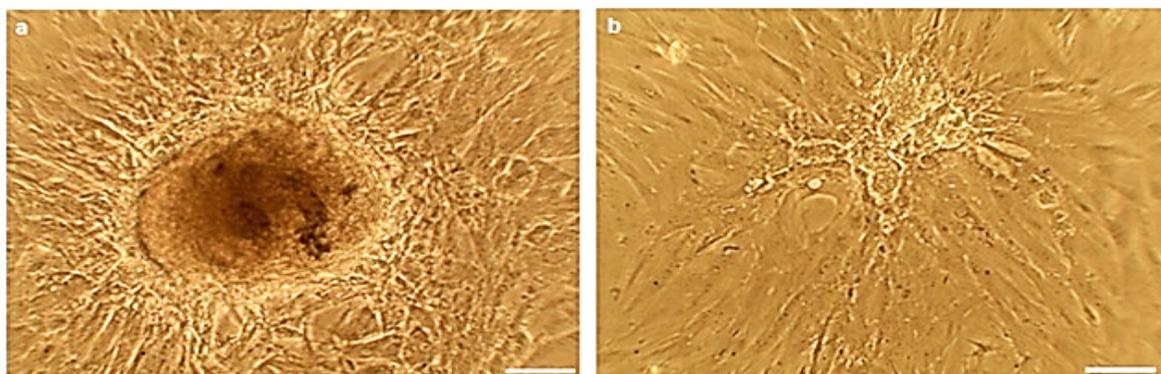
*Stra8*, *DAZL*, and *Piwill2* gene levels were higher in the experiment group than the control group (P<0.05) (Figure 4). The expression levels of the above genes in the experiment group showed an increase during culture. A significant downregulation (Figure 5) appeared in the expression of *Stra8*, *DAZL*, *Piwill2*, in the control group compared with the experiment group during two weeks culture (P<0.05) (Figure 6).

## Discussion

We designed an efficient culture system that is similar to testicular niche and may improve the viability and colony formation (as a marker of SSCs proliferation). Our system amplified the specific level and alteration pattern of spermatogonia stem cell genes which are effective in the enrichment of SSCs propagation.

The results showed that in the experiment group, the number and diameters of SSC colonies were higher than those in the control group. This is due to the stimulatory effects of interstitial cells via their interactions with SSCs in vitro culture. These results are consistent with previous studies that reported the overexpression of some specific markers of SSCs in the presence of Sertoli cells [14-16].

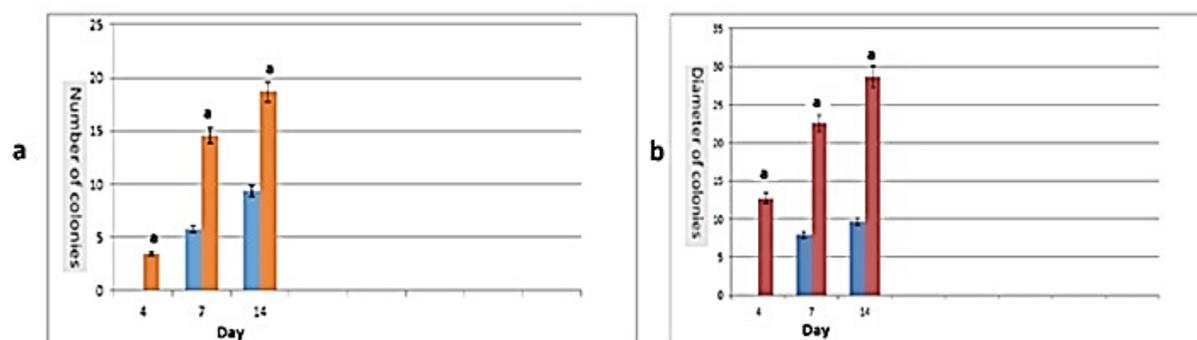
The stimulatory effect of interstitial cells including the Sertoli cells on the survival rate and proliferation of SSCs is related to the secretory activity of interstitial



**Figure 1.** The appearance of colonies derived from SSCs

A: Colonies appearance in the experiment; and B: Cultured spermatogonial stem cells in the control group;

Scale bars = 60  $\mu$ m



**Figure 2.** The number and size of SSC



A: The comparison between the number; and B. Diameter of SSC colonies at the co-culture group compared with the control group in days 4, 7, 14 of culture

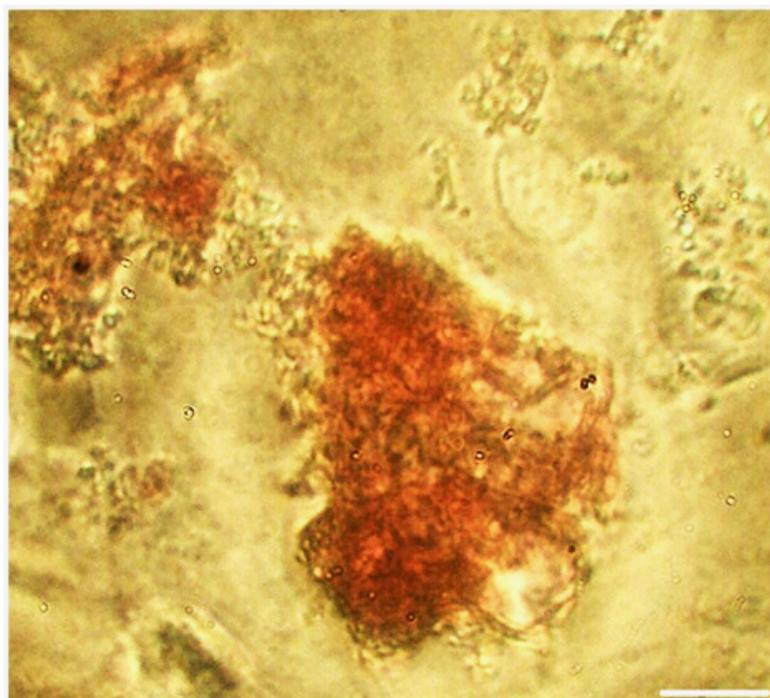
A: Significant increase with the control group on the same day ( $P < 0.05$ ).

cells and the production of various essential growth factors, especially GDNF [17, 18]. We revealed the ALP activity of SSCs colonies that were cultured with interstitial cells and detected high ALP reaction during culture. However, the ALP reactivity showed no change in the control group. Low level ALP activity after the deletion of Sertoli cells confirms their regulatory role for preservation of SSCs [19].

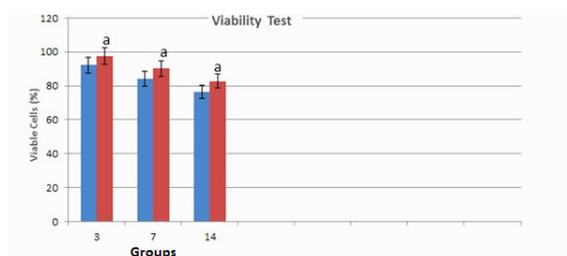
The molecular assessments exhibited that the specific genes were significantly higher in the experiment group

compared with the control group. Our data revealed a significant increase in *DAZL*, *Stra8*, *Piwill2* genes in the experiment group during the culture period. It seems that using the testicular cells in culture condition supports the stemness profile of SSCs [19-21]. It is concluded that the presence of interstitial cells in the culture regulates the natural niche factors that are essential for the maintenance and proliferation of SSCs [20].

Enrichment and improvement of spermatogonial culture condition result in a sufficient propagation before



**Figure 3.** Alkaline phosphates staining of SSCs colonies in the experiment group that shows a positive reaction to ALP



**Figure 4.** Viability in co-culture group compared with the control group in days 4, 7, 14 of culture

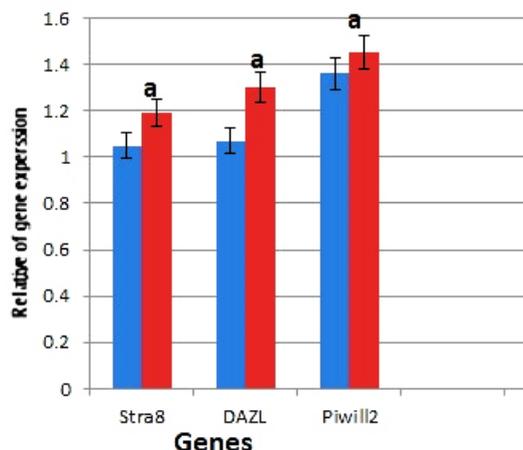
A: Significant increase with the control group on the same day ( $P < 0.05$ ).

transplantation and increase the efficiency of treatment of infertility with male factor.

Previous reports have revealed the positive effects of various growth factors such as

GDNF, LIF, bFGF, and FGF on expand them [21-23]. It seems that Sertoli cells support the SSCs proliferation via the upregulation of some specific spermatogonia markers resulting in the enrichment of SSCs. The natural signals derived from testicular niche have the determinative effects on spermatogonia stem cells proliferation and differentiation [24, 25].

This study confirms the potential application of interstitial cells as a supporting layer for SSCs propagation that has a beneficial effect for infertile men. More investigation is needed before using this method in human reproductive therapy.



**Figure 5.** The comparison of ratio genes of *Stra8*, *DAZL*, and *Piwill2* (specific spermatogonial markers)

In the experiment to the control group, after 2 weeks of culture;

A: Significant increase ( $P < 0.05$ ).

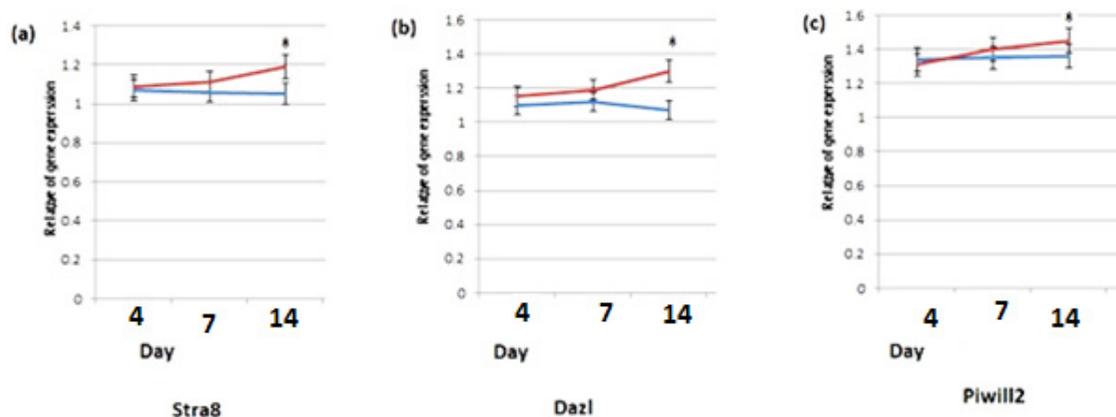
### Conclusion

This research showed that providing a similar micro-environment to testicular niche leads to an increase in viability and colonization of SSCs and regulates the expression of specific spermatogonial stem cell markers.

### Ethical Considerations

#### Compliance with ethical guidelines

The study protocol followed the rules and regulations set by the experimental animal Ethics Committee of Faculty of Medical Sciences, University of Mazandaran.



**Figure 6.** The comparison of ratio genes of *Stra8*

A: *DAZL*; B: *Piwill2*; C: In the experiment group with the control group, during 2 weeks culture;

\* Significant increase in the gene expression level ( $P < 0.05$ ).

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## Authors' contributions

All authors were equally contributed in preparing this article.

## Conflict of interest

The authors declared no conflict of interest.

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