Narimanpour *et al.*
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RESEARCH

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Spermatogenic differentiation of spermatogonial stem cells on three-dimensional silk nanofiber scaffold

Zeinab Narimanpour[1], Maryam Nazm Bojnordi[1,2*] and Hatef Ghasemi Hamidabadi[1,2]

Abstract

Background: Nano-fibrous scaffolds provide a three-dimensional matrix that guides sufficient orientation of seeded cells similar to a natural niche. In this research, we designed a silk scaffold to improve the differention of mouse spermatogonial stem cells to spermatogenic cell lines. Spermatogonial stem cells were collected from neonatal mouse (2–6 days) testes (n=60) using a two steps mechanical and enzymatic method. Cells were seeded on a silk scaffold and were cultured in Dulbecco's modified Eagle's medium, supplemented with 15 % fetal bovine serum and 1000 units/ml leukemia inhibitory factor, and incubated at 32°C in a humidified atmosphere of 5% CO_2 in air. SEM technique was done for confirmation of seeding cells.

In this study two major groups (i.e., 2D and 3D culture groups) of 30 mice each. Isolated testicular cells from each group were cultured in the absence of silk scaffold or the presence of silk scaffold.

For induction of differentiation, seeded cells on a scaffold were exposed to 1 μM and 50 ng/ml BMP-4. The specific spermatogenic genes, e.g.; VASA, DAZL, PLZF, and Piwil2, were assessed via real-time PCR and immunocytochemistry techniques. P values less than 0.05 were assumed significant. All experiments were performed at least three times.

Results: SEM analysis confirmed the homogeneity of fabricated silk scaffold and average diameter of 450 nm for nanofibers fibers. Silk scaffold induces attachment of SSCs in comparison to the monolayer group. Spermatogonia stem cell colonies were observed gradually after 1 week of culture. Electrospun scaffold supports the differentiation of SSCs to spermatogenic lines. Dates of real-time PCR showed that the expression of meiotic markers, VASA, DAZL, and Piwil2 as related to specific spermatogenic genes, had a significant upregulation in cell-seeded silk scaffold compared to the control group (P < 0.05).

Immunocytochemistry founding approved the expression of specific spermatogenic markers; DAZL and PLZF were higher in the experiment group compared to the control (P < 0.05).

Conclusion: It is concluded silk scaffold induces spermatogenic differentiation of mouse spermatogonial stem cells in vitro.

Keywords: Differentiation, Scaffold, Silk, Spermatogonia, Stem cells

Background

Three-dimensional culture system is a usable method for induction of the spermatogenesis process and treatment of male infertility [1].

In vitro spermatogenesis is a new effective therapeutic strategy for male infertility. To this aim, convenient culture systems seemed to be essential that are similar

*Correspondence: bojnordim@yahoo.com

[2] Immunogenetic Research Center, Department of Anatomy & Cell Biology, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran
Full list of author information is available at the end of the article

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to the native microenvironment of spermatogonia stem cells. In fact, the testicular niche is an essential element for the differentiation of SSCs to spermatogenic cell lines [2, 3].

Despite two-dimensional cells based on culture systems without metabolic and proliferative gradients, three-dimensional cultures are similar to the natural SSC niche and improve cell proliferation and differentiation of SSCs.

The combination of the scaffolds with stem cells may have a therapeutic application in tissue engineering. Up to now, natural polymers are biodegradable biomaterials usable as scaffolds in tissue engineering. Also, electrospinning is a suitable strategy for the fabrication of nanofibrous scaffolds with large surface areas for cell attachment. Previous studies indicated the importance of fiber diameter for cell attachment and outgrowth.

We used silk nanofiber as natural scaffolds for SSCs differentiation into spermatogenic lines.

Fabrication of nano-fibrous scaffolds mimics extracellular matrix leads to cell attachment and differentiation. Natural polymers such as silk are developed for the cultivation, proliferation, and differentiation of stem cells [4, 5].

We hypothesized electrospun silk scaffold could improve the spermatogenic differentiation of mouse spermatogonia stem cells. Therefore, we explore the effect of nanofibers silk scaffold on the differentiation of mouse spermatogonial stem cells.

Materials and methods

Fabrication of electrospun silk nanofibers

The electrospun silk nanofibers were prepared according to the procedure of Xu et al. [6]. SEM was used for the morphology of the scaffold.

Spermatogonia stem cells isolation

The experimental research was conducted after approving to the guidelines of the Animal Ethics Committee of the Medical University of Mazandaran (IR.MAZUMS..REC.1397.1817). The testes of the neonatal mouse (2–6 days) (n=60) were collected according to the previous procedure [7]. Briefly, after digesting the testis using the mechanical and enzymatic method, the cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco), supplemented with 15% fetal bovine serum (FBS; Invitrogen) and 1000 units/ml leukemia inhibitory factor (Sigma-Aldrich). In this study, there are two major groups (i.e., 2D and 3D culture groups) of 30 mice each. Isolated testicular cells from each group were cultured in the absence of silk scaffold or the presence of silk scaffold.

Cells seeding and culture

Spermatogonia stem cells (~2×10^4cells) were seeded on a silk scaffold following sterilization with UV and then were cultured in DMEM and incubated at 32°C in a humidified atmosphere of 5% CO_2 in air [8].

Scanning electron microscopy

SEM technique was done for the attachment of seeded cells on scaffold. After washing, cells were fixed with glutaraldehyde and paraformaldehyde for 90 min, then dehydration was done and dried at room temperature and assessed with SEM.

In vitro differentiation of SSCs

In order to evaluate the effect of silk scaffold on the differentiation of SSCs to the spermatogenic line, cultured SSCs were induced to differentiate by RA and BMP-4 in the presence (3D group) and absence (2D or control group) of silk scaffold. For the induction of differentiation of the SSCs, cells were exposed to RA (1 μM RA, Sigma-Aldrich) and BMP-4 (50 ng/ml BMP4, Sigma-Aldrich).

Quantitative real-time PCR

Total RNA from cultured cells was extracted using the RNeasy Micro kit (Qiagen, Hilden, Germany). cDNA was synthesized via QuantiTect Reverse Transcription Kit (Qiagen) from ~1 μg of extracted RNA per the manufacturer's instructions. Q-PCR was carried out using Master Mix and SYBR Green in a thermocycler. and PCR program includes an initial denaturation step of 95°C for 5 min, followed by 40 cycles of melting (30 s at 95°C), specific annealing (40 s at 55°C), and extension (30 s at 72°C). The comparative CT (cycle threshold) method was done for the ratio of gene expression and the comparative CT method ($2^{\Delta\Delta CT}$) was done for the relative quantification of genes. βactin normalized used as a housekeeping gene [9].

Immunocytochemistry

Cells were fixed in paraformaldehyde for 20 min and incubated in goat serum 5% (Sigma) for 30 min. then exposure to monoclonal antibody DAZL (abcam) (1:200), and monoclonal antibody PLZF (abcam) (1:300) overnight. Secondary antibodies were added for 1 h. After staining with 4,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) (1:1000).

Statistical analysis

Data was analyzed using a one-way analysis of variance and T test followed by Tukey's post hoc test. Data are given as means ± standard deviation. P values less than

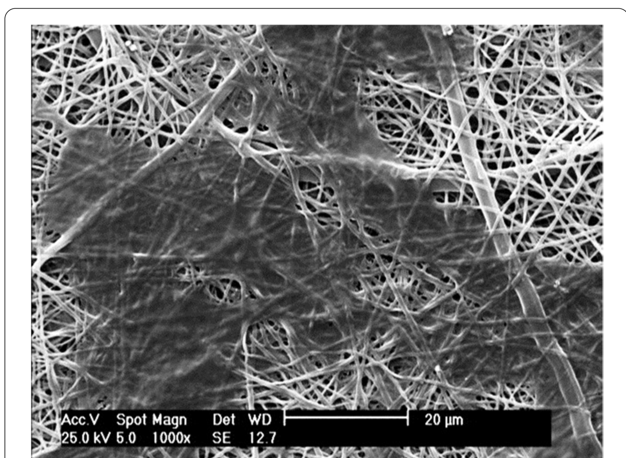

Fig. 1 Scanning electron microscopy micrographs of SSCs seeded on silk scaffolds. SEM analysis confirmed interaction and attachment of grown SSCs in silk scaffold after 5 days of culture

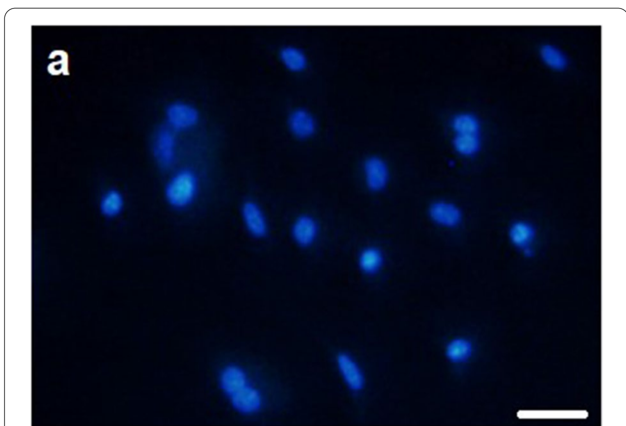

Fig. 2 Immunofluorescent graph of grown cells on silk scaffold. Spermatogonia stem cells were grown on the silk scaffold stained with DAPI

0.05 were assumed significant. All experiments were performed at least three times.

Results

SEM analysis

We used electrospinning technique for Silk nanofibers fabrication. Size and morphology of the nanofibers scaffolds were tested using the SEM technique. SEM analysis confirmed the homogeneity of fabricated silk scaffold without any bead fibrous structure and branching. The average diameter of 450 nm for nanofibers fibers. Also, cell attachment was confirmed via SEM micrographs (Fig. 1).

SEM analysis confirmed interaction of grown SSCs in Silk scaffold after 5 days of culture. Seeded SSCs were attached firmly on a silk scaffold. The attached cells on the scaffold were stained with DAPI (Fig. 2). Also, Spermatogonia stem cells were monitored daily using a phase-contrast microscope morphologically. Some SSCs colonies were observed gradually after 1 week of culture. (Fig. 3).

Real-time PCR

In order to evaluate the effect of silk scaffold on the differentiation of SSCs to the spermatogenic line, cultured SSCs were induced to differentiate by RA and BMP-4 in the presence (3D group) and absence (2D or control group) of silk scaffold. The identity of induced cells was finally determined by quantitative expression analysis of spermatogenic markers at the mRNA level.

The capability of silk scaffold for the induction of differentiated SSCs was explored by quantifying the expression levels of VASA, DAZL, and Piwil2 which are pre-meiotic markers for differentiation of SSCs to spermatogenic lines.

Expression levels of pre-meiotic markers before differentiation induction and the potential of silk scaffold for differentiation of SSCs to spermatogenic lines were explored via the expression levels of VASA, DAZL, and Piwil2 as related to specific spermatogenic genes. After

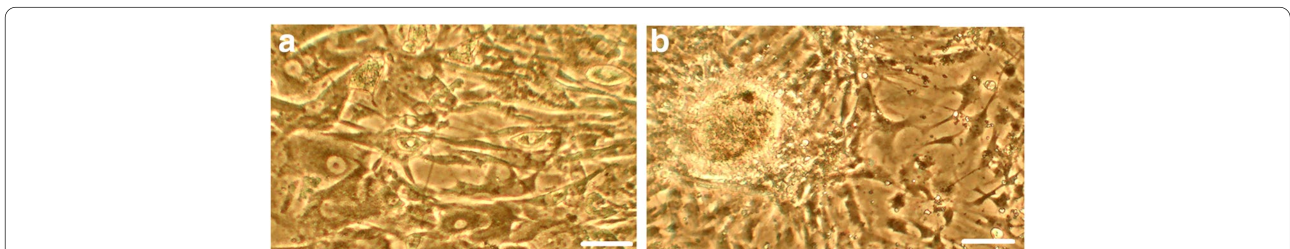

Fig. 3 The morphology of spermatogonial stem cells during culture. SSCs appearance on the 5th day (a) derived SSCs colonies' 14th day of culture. Scale bars 100 mm

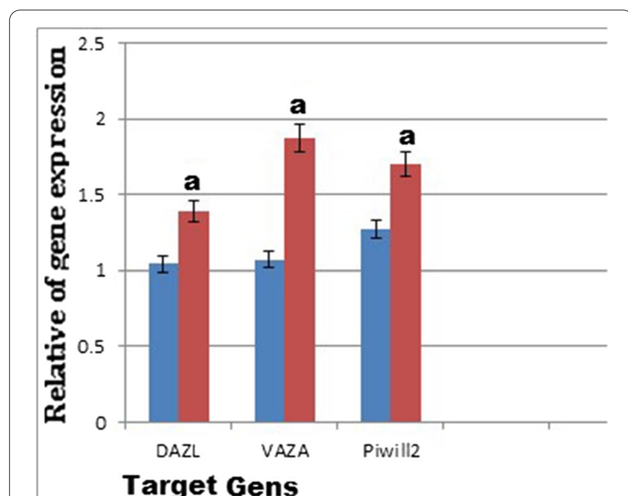

Fig. 4 Comparison of specific spermatogonia gene expression, in seeded SSCs on Silk scaffolds. Ratio gene expression of DAZL, VASA, and Piwil2 in SSCs grown on scaffolds *compared to control*. Data are shown as mean±SEM from three independent experiments. a: significant increase with the control group

2 weeks of culture, Expression of these meiotic markers showed upregulation in cell-seeded on silk scaffold compared to the control group (Fig. 4). Three replicate analyses were carried out for each culture group. All PCR reactions were performed at least in triplicate.

Immunocytochemistry

Specific spermatogonial markers were assessed via immunocytochemistry technique. The expression of specific spermatogenic markers, DAZL and PLZF, was higher in the experiment group compared to the control (Fig. 5).

Discussion

SSCs has potential for differentiation to male gametes, e.g., spermatozoid that transfer genetic information to the next generation [10, 11].

Stem cell therapy based on SSCs transplantation is applicable in the treatment of infertility.

Previous researches showed that a three-dimensional culture system is a usable method for induction of the spermatogenesis process and treatment of male infertility [12, 13]. In fact, in vitro spermatogenesis is a novel treatment for male infertility.

Combination of the scaffolds with stem cells is convenient for culture systems similar to the native testicular microenvironment. This microenvironment has a crucial role in SSCs differentiation to spermatogenic cell lines [14, 15].

Also natural polymers for the fabrication of scaffolds in tissue engineering. So in this research, we evaluate the effect of an electrospun silk scaffold on the differentiation of spermatogenic lines. Our results approved a promising performance for silk scaffold in supporting of SSCs towards differentiated spermatogenic cells [16].

Similar research has declared the proliferation and differentiation of spermatogonia stem cells using a soft agar culture system similar to in vivo conditions [17, 18].

Our results are in line with the findings of other previous studies. In a previous study, 3D culture system increased spermatogonia stem cell colonies' number and diameter as well as the maturation of pre-meiotic compared to a two-dimensional culture system [19, 20].

We used RA and BMP-4 as inducers for SSCs differentiation that promote differentiation of SSCs and meiosis division [21, 22].

Our dates showed the up-regulation of VAZA, DAZL, and Piwill2 genes in experiment groups at the end of the spermatogenic induction (on the 28th day of the experiment).

Immunocytochemistry findings proved the expression of PLZF and DAZL in cells grown in silk scaffolds system. In total, it is concluded that culturing of SSCs exposure to inducers RA and BMP-4 and in a 3D culture system stimulated differentiation of SSCS [23–25].

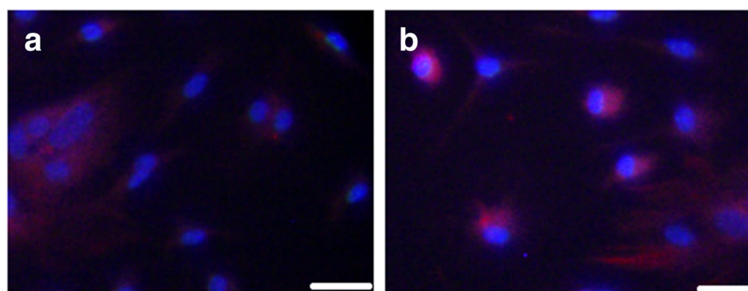

Fig. 5 Immunoflourcent staining of differentiated cells seeded on scaffold. The differentiated cells were immunopositive to specific spermatogenic markers of DAZL (**a**) and PLZF(**b**). scale bars, 100 µ

VAZA, Protamin, and DAZL are meiotic markers that are expressed during the meiotic stage [26–28].

These markers were expressed significantly in the cells cultured on scaffolds in comparison to the monolayer group.

In this study, we proved spermatogonia stem cells grown on silk scaffold induce cell differentiation in vitro compared to monolayer groups. Results of this research emphasize the importance of 3D culture system applications that confirms our research describing a successive maturation of meiotic SSCs in the culture system.

Our findings mimic some aspects of the natural three-dimensional microenvironment to differentiation of SSCs to spermatogenic lines in vitro.

This can be usable as a novel culture system for differentiation of spermatogonia stem cells to spermatogenic cell lines with an applicable therapeutic approach for infertile men.

Availability of data and materials

The datasets are available from the corresponding author on reasonable request.

Abbreviations

SSCs: Spermatogonial stem cells; RA: Retinoic acid; BMP-4: Bone morphogenetic protein 4; DMEM: Dulbecco's modified Eagle's medium; ECM: Extracellular matrix; SEM: Scanning electron microscopy; DAZL: Deleted in azoospermia like; PLZF: Promyelocytic leukemia zinc finger protein; Piwil2: Piwi-like protein 2; PCR: Polymerase chain reaction; MTT: Methylthiazolte- trazolium; DAPI: 4′, 6-diamidino-2-phenylindole; cDNA: Complementary DNA; 3D: Three dimensional; FBS: Fetal bovine serum.

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Authors' contributions

MNB contributed to the design, experimental test, and drafting of the manuscript. HGH contributed to the formal analysis, statistical analysis, and editing of the manuscript. ZN contributed to the methodology and experimental test. The authors have read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

All procedures after approved by the animal ethical committee of Mazandaran medical science university in accordance with the ethical standards of the institutional and/or national research committee. Approval for the research was given by the Ethics Committee at the Mazandaran medical science university (IR.MAZUMS..REC.1397.1817).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

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

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