Iranian Journal of Reproductive Medicine Vol.5. No.3. pp:109-115, Summer 2007

Colony formation ability of frozen thawed spermatogonial stem cell from adult mouse

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Received: 1 June 2007;accepted: 6 September 2007

Abstract

Background: The basis of spermatogenesis is the spermatogonial stem cells (SSCs). The concentration of SSCs is very small. However, a system that supports the proliferation and maintenance of SSCs in vitro could be used to preserve and expand SSCs numbers as well as increase success in transplantation. It is a new avenue to restore spermatogenesis in azoospermia subjects.

Objective: Proliferation and enhancement of frozen-thawed SSCs numbers during in vitro culture**.**

Materials and Methods: Both Sertoli and spermatogonial cells were isolated from adult mouse testes. Frozen-thawed spermatogonial cells were cultured in two groups: simple culture (Experimental 1) and co culture with Sertoli cells (Experimental 2). Also, Fresh cells were considered as control groups: simple culture (control1) and co culture with Sertoli cells (control 2).Assay of the spermatogonial-cell-derived colonies was carried out at the end of each week.

Results: Results indicated that the viability rate of the frozen cells after thawing (68.4±10.2%) was influenced by cryopreservation procedure significantly ($p \leq 0.001$). In addition, the number of the colonies and their diameters in the co-culture system with fresh cells (25.1±5.2 and 205.8±50 µm, respectively) were more than other groups and the differences were significant ($p<0.001$). Number of the colonies and their diameters in experimental 1(9.5±4.3 and 124±35.9 µm, respectively), experimental 2 (15.6±3.5 and 157.6±41.9µm, respectively) groups were better than control 1 group (3.1±2.2 and 87.5±30.6µm, respectively) and the differences were significant ($p<0.001$).

Conclusion: We demonstrated that co-culture system with Sertoli cells can increase in vitro colony formation of adult fresh and frozen-thawed spermatogonial cells in mouse.

Key Words: *Co-culture system, Spermatogonia, Cryopreservation, Mouse.*

Introduction

Spermatogenesis is a highly organized, complex process that relies on self-replication of undifferentiated SSCs and production of differentiated daughter cells to provide a continual supply of spermatozoa (1). Stem cells are generally defined as colonogenic cells capable of both self-renewal and differentiation in vivo (2, 3). In the adult mouse testis, there are about 35.000 stem cells which is 0.03% of all germ cells (4). However, when spermatogonial cells are cultured in suitable condition, some SSCs survive long-term culture spermatogonial and repopulate recipient testes after transplantation (5).

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Antimitotic chemotherapy or radiotherapy can induce failure spermatogenesis. Clinically, spermatogonial stem cell culture and cryopreservation in combination with auto transplantation could serve to restore male fertility after an insult to the testis. An alternative and probably the best method for long-term preservation of SSCs is cryopreservation in combination with culture. Increasing the number of frozen-thawed adult SSCs in vitro is benefit for success in transplantation specially to rescue fertility of cancer patients (6).

To date, various systems have been set up for spermatogonia of various species. Co-culture with Sertoli cell has been demonstrated to support the survival of isolated SSCs from neonate (7, 8) and adult mice testis (5). Izadyar et al (2003) developed a immature bovine spermatogonial-Sertoli cell co-culture system that allowed survival, proliferation and differentiation up to cells showing characteristics of spermatids, during at least 4 months (9). In these cultures, two types of spermatogonial colonies were formed, one consisting of only single stem cells and a mixed type in which, besides stem cells, also pairs and chains of cells were formed. Although these culture systems supported the survival and proliferation of prepubertal type-A spermatogonia greatly in vivo, proliferation was less frequently reported in adult in vitro.

The objective of the current study was proliferation and enhancement of frozen-thawed SSCs numbers during in vitro culture. To accomplish this objective, we developed a Sertoli cells monolayer for co-culture with frozen-thawed adult mice germ cells. We hypothesized that SSC colony formation could be sustained in this system and it could be enhanced by co-culture with Sertoli cells.

Materials and methods

Experimental animals

Male adult mice (age= 6-8 week; *n*=20) from the National Medical Research Institute (NMRI), initially derived from original stocks obtained from Razi Laboratory (Tehran, Iran), were maintained under standard conditions with free access to food and water at the Animal Facilities of Tarbiat Modares University (Tehran, Iran). The research was conducted in accordance with the National Research Council guidelines.

Spermatogonial cell collection

Bilateral testes were collected from adult 6-8 weeks old NMRI mice for cell suspension. They were placed on ice and transferred to the laboratory within 10 min. After decapsulation, the testes were minced into small pieces and suspended in Dulbecco's Modified Eagle medium (DMEM; Gibco, Paisley, UK), supplemented with 13.5 gr/L $NaHCO_3$ (Sigma, St Louis,MO, USA), single-strength non-essential amino acids, 100 $IUmL^{-1}$ penicillin, 100μgmL^{-1} streptomycin and 40μgmL^{-1} gentamycin (all from Gibco). The minced pieces of testis were suspended in DMEM, which contained 0.5 $mgmL^{-1}$ collagenase/dispase, 0.5 $mgmL^{-1}$ trypsin and 0.08 $mgmL^{-1}$ DNase, for 30 min (with shaking and a little pipetting) at 37°C. All the enzymes were purchased from Sigma. After three washes in DMEM medium and removal of most of the interstitial cells, spermatozoa and some spermatids cells, a second digestion step (45 min at 32°C) was performed in DMEM by adding fresh enzymes to the seminiferous cord fragments. Most of the cell aggregates that remained after this treatment were sheared gently by repeated pipetting with a Pasteur pipette for 5 min. The cells were separated from the remaining tubule fragments by centrifugation at 30g for 2 min at 37°C. After filtration through a 70-µm nylon filter, the collected cells were used for spermatocytes cells isolation. The spermatocytes cells were isolated using a procedure described by van Pelt et al. with some modifications (10). The Sertoli cells were isolated using a procedure described by Scarpino et al. with some modification (11) and overnight differential plating. After the spermatocytes cells and Sertoli cells had been isolated, the spermatogonia that remained in suspension were collected and then cultured or cryopreserved.

Spermatocytes cells isolation by PNA binding

Petri dishes with a diameter of 60 mm were coated with 5 ml, 100µg/ml PNA in PBS^+ for at least 1 h at 37°C. Then, dishes were washed three times with DMEM containing 0.5% BSA. The dishes were stored with DMEM containing 5 µg/ml DNase for at least 1h at 37°C. Cells were incubated in these dishes for 1.5 h at 32°C in an atmosphere of 5% CO_2 in air. After the binding to PNA, nonbinding cells were collected by repeated washing of the dishes with a pipette.

Sertoli cells isolation by DSA-lectin binding

Sertoli cells were extracted from adult mouse testis following the second enzymatic digestion. Briefly, the Petri dishes with a diameter of 60 mm or flask were coated with a solution of 5μgmL^{-1} of Datura stramonium agglutinin (DSA; Sigma) in

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phosphate-buffered saline (PBS) at 37°C for 1h. Then, coated plastic dishes were washed three times with DMEM containing 0.5% BSA (BSA; Sigma). The mixed population of the cells obtained by enzymatic digestion was placed on lectin-coated dishes and incubated for 1 h at 32°C in a humidified atmosphere of 5% CO_2 in air. After the incubation, the non-adhering cells were collected by being washed twice with medium. Alternatively, 4 days after the Sertoli cells formed a confluent layer, the Sertoli cells were detached by ethylenediamine tetra acetic acid (EDTA) – trypsin treatment (0.02% EDTA–0.1% trypsin in Ca^{2+}- and Mg-free PBS) for 5 min at 37°C, counted and adjusted to desired densities into petri dish for secondary culture in DMEM at 32°C in the presence of 10% fetal bovine serum (FBS; Gibco). This method helped preparation of the Sertoli cells with more than 95% purity.

Cryopreservation and thawing procedure

The isolated cells were cryopreserved using a procedure described by Izadyar et al. with some modification (12). Immediately after cell isolation, cell viability was assessed. Cell suspensions in 0.5-ml aliquots (6×10^6 cells per mL) were prepared. Then, an equal volume of 2× concentrated freezing medium was added dropwise to the Eppendorf vial containing the cell suspension during a period of 10–15 minutes, and after gently mixing by inverting the vial, a sample was taken for viability assessment. The freezing media were based on DMEM supplemented with 10% (v/v) FCS, 1.4M DMSO and 0.07 M sucrose; For noncontrolled-rate freezing, 1.8-mL cryovials vials (Nunc, Denmark) containing 1.0 mL of cell suspension in freezing medium were placed in an insulated (polystyrene) container at -80°C for at least 1 day and then plunged into liquid nitrogen. The cells were thawed by swirling in 38°C water bath for 2 minutes. The contents of the vial was transferred to a tube and diluted slowly by adding two volumes, dropwise, of DMEM supplemented with 10% FCS. Then, the cells were pooled and centrifuged at 2000 × g for 5 minutes, the supernatant was removed, and the pellet was resuspended in DMEM/FCS. A sample was taken for viability assessment, and the remainders of the cells were used for culture experiments.

Fresh and cryopreserved spermatogonial cells co-culture with sertoli cells

A monolayer of Sertoli cells were used to provide an environment that resembles that in vivo as closely as possible. Adult fresh Sertoli cells were used in a co culture system with cryopreserved or fresh adult mouse germ cells.

After formation a Sertoli cells confluent layer, frozen-thawed spermatogonial cells were cultured in two groups: simple culture (Experimental 1) and co culture with Sertoli cells (Experimental 2). In addition, Fresh cells were considered as control groups: simple culture (control1) and co culture with Sertoli cells (control 2). Cells were grown for 3 weeks. At the end of the first week, the cells were passaged and cultured for two weeks. The diameter and the number of colonies were determined every 7th days during the culture for 3 weeks.

Colony assay

Assay of the spermatogonial-cell-derived colonies was carried out in the 7th days. At the end of the first week, the cells were passaged and colony assay was carried out at 14 and 21 days during culture. An inverted microscope (Zeiss, Germany) was used to determine the number of the colonies and their diameters were being measured using ocular grid which was occupied on the inverted microscope.

Immunohistochemistry for confirmation of sertoli and spermatogonia cells

Vimentin was detected by the procedure which was described by Anway et al. in Sertoli cells (13). For an oct-4 immunohistochemistry of the obtained colonies, the colonies grown on the glass slides were stained. Oct-4 immunohistochemistry has been described as a marker for undifferentiated cells (14). Alkaline phosphatase activity was detected by the procedure of Palombi et al (15).

Statistical analysis

Results are expressed in mean±S.D. The statistical significance between mean values was determined by two and one way analysis variance (Tuky-test) in fresh and cryopreserved spermatogonial cells co-culture with Sertoli cells. $p \leq 0.05$ was considered significant.

Results

Isolation and characterization of spermatogonial and Sertoli cells

The cells population obtained from DSA-lectin isolation proliferated and created a monolayer of cells (Figure 1.A). They had an irregular outline with a granular appearance. The monolayer cells showed no alkaline phosphatase reactivity (Figure

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1.D). Meanwhile, the control groups, consisting of mouse intestine (brush border of villous), adult mouse testes (the endothelial cells, smooth muscle cells of the blood capillaries in the interstitium and peritubular myoid cells) showed alkaline phosphatase reactivity (Figure 1B, C). Moreover, Vimentin, which is a molecular marker for Sertoli cells, was detected in the feeder monolayer cells (Figure 1E, F). The other cells type, with spherical outline and two or three eccentrically placed nucleoli, created a colony after proliferation during first week or immediately after passage (Figure 2.A). The resulted colonies had clearly AP activity. Moreover, Oct-4, which is a molecular marker for SSCs, was detected in the obtained colonies (Figure 2.C, D) but c-kit wasn't detected in them.

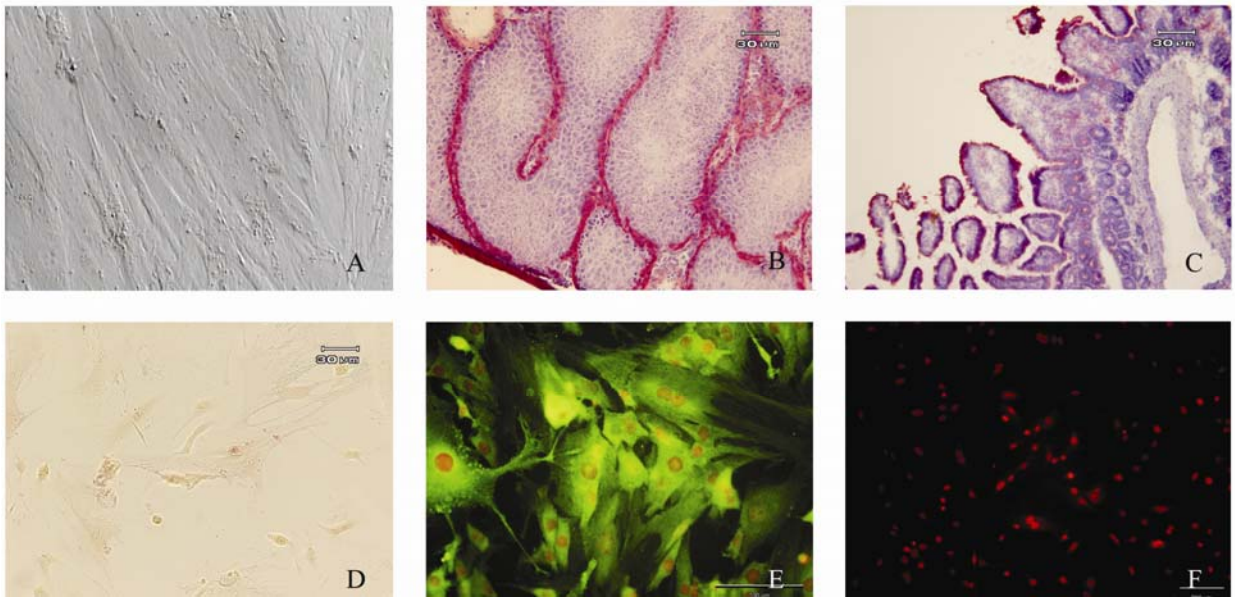


Figure 1. Sertoli cells: (A) The cells population obtained from DSA-lectin isolation proliferated and created a monolayer of cells. Alkaline phosphatase reactivity in the control groups, consisting of (B) adult mouse testis, (C) mouse intestine, (D) the monolayer cells did not showe alkaline phosphatase reactivity. (E) Vimentin was detected in the feeder monolayer cells and (F) the control group. Magnification: ×400 (in A,B,D,E); ×100 (in C, F)

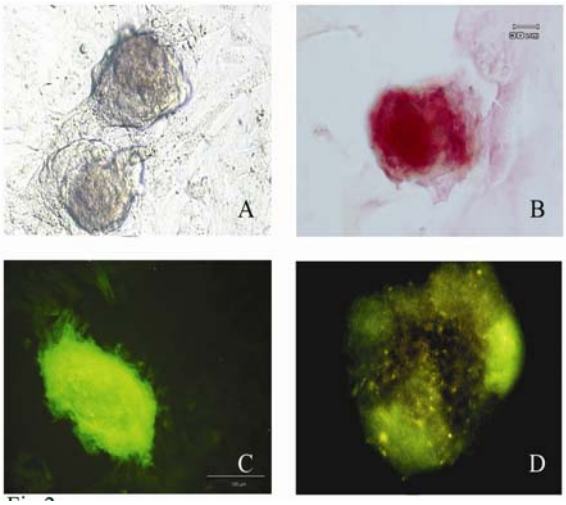


Figure 2. Spermatogonial cells: (A) The morphology of a spermatogonial-derived colony formed from co-cultured spermatogonial cells on a monolayer of Sertoli cells. (B) The colony cells showed alkaline phosphatase reactivity, (C) Oct-4 was detected in cells of colony, but c-kit was not detected. Magnification:×100 (in *A*); ×400 (in *B,C,D)*

Effect of cryopreservation on viability rate after thawing

Viability rate of cells after isolation process and after mixing by freezing media were 92.8±6.03% and 85.5±5.7% respectively. These demonstrate that freezing media does not have a significant effect on viability rate. Only 68.4±10.2% of the frozen cells survived after cryopreservation. The viability rate was influenced by the freezing and thawing procedure significantly ($p \leq 0.001$).

Colony assay in fresh and cryopreserved spermatogonial cells co-culture with Sertoli cells

Taken together, as demonstrated in Tables I and II, our results indicated that number of the colonies and their diameters in the co-culture with fresh cells (25.1±5.2 and 205.8±50 µm, respectively) were more than other groups and the differences were significant ($p<0.001$). Also, number of the colonies and their diameters in experimental 1 (9.5±4.3 and 124±35.9 µm, respectively) and experimental 2 (15.6±3.5 and 157.6±41.9µm, respectively) groups were better than control 1 group (3.1±2.2 and 87.5±30.6µm, respectively) and the differences were significant ($p<0.001$).

The colonies appeared significantly earlier (on day 4) in co-culture groups compared to colony formation time in control 1 group (on day 7 or after passage).

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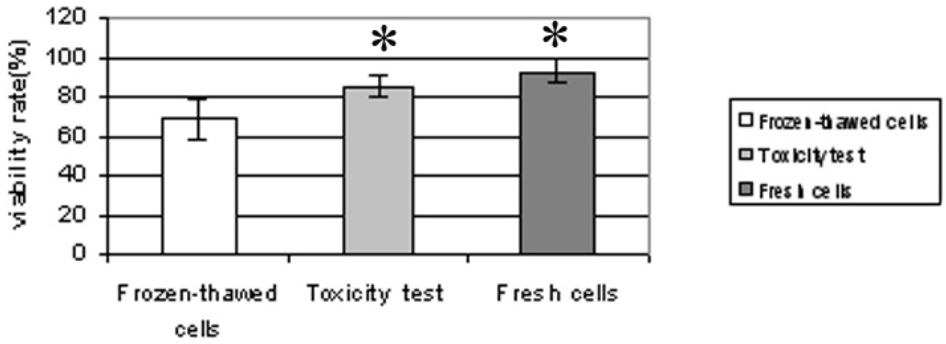


Figure 3. Comparison between the viability rates in fresh cells and frozen-thawed groups after isolating and toxicity test. *Significant difference v .frozen- thawed group ($p \leq 0.001$)

Table I. Comparison of colony number between control and experimental groups**.** Values are the mean colony numbers at different times±S.D. Results from five separate experiments were used for all groups.

Group	Week1	Week2	Week3
Control 1(fresh cell)	2.1±0.8	3.1±1.3	3.1±2.2
Control 2 (fresh cell co-culture with Sertoli cell)	28.3±5.9[a]	27.8±7.0 [a]	25.1±5.2[a]
Experimental 1 (frozen-thawed)	7.7±3.9[b]	11.5±7.1	9.5±4.3[b]
Experimental 2(frozen-thawed co-culture with Sertoli cell)	10.3±2.3[a*b]	28.6±6.7 [a bc**d]	15.6±3.5[ab*]

[a]*Significant difference v. control group in the same column(p <0.001).*
[b]*Significant difference v. Co-culture with Sertoli cell group in the same column (p <0.001).*
[c] *Significant difference v. Experimental 1 group in the same column.*
[d] *Significant difference v. week1and week3 in the same row*
**(p <0.05), ** (p <0.01)*

Table II. Comparison of colony diameters between control and experimental groups. Values are the mean colony diameters at different times ±S.D. Results from five separate experiments were used for all groups.

Group	Week1	Week2	Week3
Control 1(fresh cell)	90.8±24.5	90±22.8	95±27.4
Control 2 (fresh cell co-culture with Sertoli cell)	198.3±51.1[a]	187.9±41.9 [a]	205.8±50 [a]
Experimental 1 (frozen-thawed)	114.7±36.7[b]	123.4±33.5 [b]	124±35.9 [b]
Experimental 2(frozen-thawed Co-culture with Sertoli cell)	157.8±57.2 [a**bc**]	140.7±30.6 [ac]	157.6±41.9 [abc]

[a]*Significant difference v. control group in the same column (p <0.001).*
[b]*Significant difference v. Co-culture with Sertoli cell group in the same column (p <0.001).*
[c]*Significant difference v. experimental 1group in the same column (p <0.001).*
** (p <0.05), ** (p <0.01)*

Discussion

In this study, we demonstrated that co-culture system with Sertoli cells can increase *in vitro* colony formation of adult fresh and frozen-thawed spermatogonial cells.

For confirmation of the presence of spermatogonial cells in resulted colonies in addition to alkaline phosphatase activity assessment, c-kit and Oct-4 were traced in the colony cells as well.

Spermatogonial-derived colonies showed alkaline phosphatase activity and Oct-4 expression, but they didn't showed c-kit expression. This finding is in agreement with that reported by the previous investigators who demonstrated alkaline phosphatase activity and Oct-4 expression in the colony cells (14, 16, 17). But it is in disagreement with Richards et al (1999) who demonstrated primordial germ cells are alkaline phosphatase positive , but from the gonocyte stage onwards, the germ cells are negatively stained (18). In the mouse testis, A_s and A_{pr} spermatogonia are c-kit-negative, whereas late A_{al} to B spermatogonia are c-kit positive. Our result in c-kit expression is similar with Schrans-Stassen et al (1999) and Izadyar et al (2002) (19,20). For confirmation of the presence and purification of Sertoli cells as feeder cells, in addition to alkaline phosphatase activity assessment, specific marker detection was carried out using immunocytochemistry anti-Vimentin antibody. Feeder-monolayer did not show alkaline phosphatase activity. Vimentin is a cytoskeletal protein usually found in the epithelial cells; this protein is a marker for Day 14 postnatal Sertoli cells (11) and is strictly localized at the perinuclear region of the cells (21, 22). This finding is in agreement with the reports by

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Scarpino et al (1998) as well as Anway et al (2003) (11,13).

We demonstrated that frozen/thawed adult spermatogonial stem cells survive from freezing procedure and formed colony in culture. This finding is in agreement with that reported by Izadyar et al (2002) in perpubertal bovine(12). The viability rate of freshly isolated adult spermatogonial cells was higher than that of frozen/thawed cells. Also, the colony formation efficiency of freshly spermatogonial cells co culture with Sertoli cells was higher than that of frozen/thawed cells. This was mainly due to the diminished cell recovery following the freeze/thaw procedure. Reduced cell recovery following the freeze/thaw procedure was also reported by other investigators studying cryopreservation of non pure spermatogonia from other species, including rodents (23, 24) and domestic animals (20,25).

Number of the colonies and their diameters in cryopreserved groups were better than fresh group. Maybe it is due to the damages and shocks resulted from cryopreservation procedure in differentiated germ cells. Cryopreservation probably can causes purification and increases efficiency of colony formation in isolated spermatogonial stem cells.
Number of the colonies in all groups declined at the three weeks. Colonies probably start to develop when spermatogonia and Sertoli cells make contact, apparently creating a microenvironment that favors their development. SSC might need specific micro contacts with niche-offering Sertoli cells during culture (26). Physical contact and secreted growth factors and cytokines affect the survival of spermatogonial stem cells and provide a suitable microenvironment for proliferation and colonies formation. Previous studies have shown that Sertoli cells monolayer support germ cells proliferation and differentiation (27). Better results have been obtained with co-cultures of Sertoli cells with mouse and bovine spermatogonia (9, 26). Other recent results indicate that the presence of Sertoli cells is deleterious to stem cells probably through the induction of differentiation (28). Very likely, the decline may be the result of apoptosis or detachment of differentiating germ cells, as reported previously (26). However, the remaining survived colonies were increased in size. It seems that two week is suitable for duration culture.

Conclusuion

In summary, these findings demonstrate that spermatogonial stem cells from adult mouse testis can be successfully cryopreserved and are able to form colony after freezing and thawing procedures. Also, co-culture system with Sertoli cells can increase *in vitro* colony formation of adult fresh and frozen-thawed spermatogonial cells.

Acknowledgment

We highly appreciate the contribution of Dr. Ans van Pelt and Prof. Dr. Dirk De Rooij (Fertility Laboratory, AMC, and Amsterdam) for their consults as well as the Shahram Pour biranvand (Department of Anatomical Sciences, School of Medical Sciences, Tarbiat Modares University) and Royan Institute (Tehran, Iran) for its technical assistances. This work was supported by a research grant from Tarbiat Modares University and Iranian Stem cell Network.

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