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Short Report

Immortalization of type A spermatogonia using the SV40 Large T Antigen

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ABSTRACT The study of the *in vitro* behaviour and experimental manipulation of spermatogonia is limited due to the absence of an effective protocol for long culture of germ stem cells (Nagano *et al.,* 1998). Some years ago, Hofmann *et al.* (1992) described the possibility of inducing immortalization of germ cells by transfection of the SV40 large T antigen. Nevertheless, these authors only produced continuous type B spermatogonia cell lines and other somatic cells of the testis. In this communication we describe a modification of their method that can be used to generate continuous cell lines of the most undifferentiated germ cells of the testis, that is type A spermatogonia.

Introduction

Hofmann *et al.* described in 1992 the use of the SV40 large T antigen to immortalize germ and somatic cells of the testis. These authors isolated four cell lines using discontinuous Percoll gradient centrifugation. The cell lines were originated from 10.5 days old animals and corresponded to peritubular fibroblastic cells, Leydig cells, Sertoli cells and type B spermatogonia. In our study, we modified the protocol used by these authors to obtain a pure population of immortalized type A spermatogonia.

Materials and Methods

Spermatogonia purification

Tunica albuginea of 7.5 day old C57/BL6 mice testis were removed and the content of the testis was incubated with collagenase II (C-6885, Sigma) in a concentration of 1mg/mL in Hank´s Balanced Salt Solution (H-8264, Sigma) at 37 ºC over 5 minutes to isolate seminiferous tubuli. Then the isolated seminiferous tubules were centrifuged at low speed and resuspended the seminiferous tubuli in trypsin-EDTA 0,05%-0,02% (25300-054 Gibco) over 5 minutes at 37 ºC. The testicular cell suspension was filtered through a nylon net with pores of 40 micrometers(2340 Falcon). Filtered suspension was centrifuged again at 390 g over 10 minutes. Pellet was resuspended in culture medium and seeded in culture flasks.

Culture conditions

Culture medium was Dulbecco´s modified Eagle´s medium (D-6421, Sigma) containing 10% foetal bovine serum with 6 mM glutamine (G-6392, Sigma), 1% sodium piruvate (P-4562, Sigma), 0.1% non essential amino acids (M-7145, Sigma) and 1% nucleosides (G-6264, A-4036, C-4654, T-1895, U-3003, Sigma) and 0.5 % penicillin-streptomycin (P-0781, Sigma) added. Culture conditions were 37 ºC in an atmosphere of 5% carbon dioxide in air. After 12 hours in culture, Sertoli cells appeared attached on the button surface of the flask, while spermatogonia remained in the supernatant.

Electroporation

Type A spermatogonia were removed from the flask, centrifuged at 390 g over 5 minutes and resuspended them in 800 μL of PBS. We added 10 ng of the pL575 plasmid to 10^6 cells. Cells were maintained in the electroporation cuvette (1652088, BioRad) on ice for 10 minutes. Then, we applied a high voltage electrical pulse at 900 kV and 50 micro faradays during 1.4 milliseconds (Gene Pulser II, BioRad). After electroporation, the cuvette with the cells was incubated on ice for 20 minutes and then, spermatogonia were seeded on the feeder layer of Sertoli cells.

The expression of c-kit antigen was tested by immunofluorescence (MCA1365, Serotec).

Results and Conclusions

The main differences between the protocol of Hoffman *et al.* and ours are the following:

1- We use 7.5 day old mice because in this stage there is a pure population of type A spermatogonia in the testis (Romrell *et al.,* 1976).
2- We separate type A spermatogonia from somatic cells thanks to their differential plastic adhesion properties. While Sertoli cells are adherent to the wall of the flask, type A spermatogonia remain alive and floating in the culture media for about 12 hours.
3- Once we obtained the pure population of type A spermatogonia, we do electroporation transfecting the SV40 large T antigen, instead the calcium phosphate procedure used by Hofmann *et al.* (1992).
4- Electroporated cells were seeded over a feeder layer of a primary culture of Sertoli cells because the maintenance *in vitro* of spermatogonia benefits from coculture (Brinster *et al.,* 1998).
5- Type A spermatogonia can be recognized using an indirect immunostaining with an anti c-kit antibody (Dym *et al.,* 1995).

The colonies of type A spermatogonia survived more than five weeks in our laboratory.

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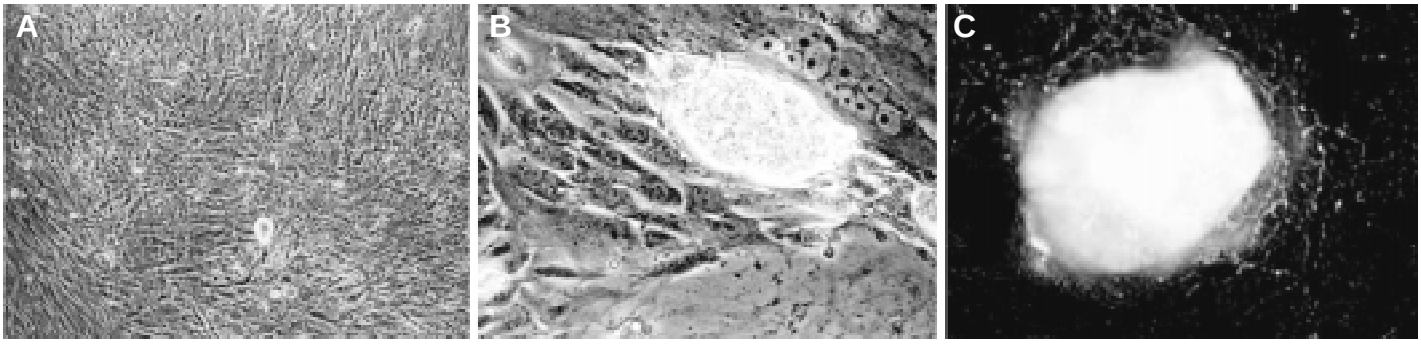

Fig.1. Cultures of type A spermatogonia with or without SV40 large T antigen electroporation. (A) *Five week culture of type A spermatogonia over Sertoli cells as feeder layer without being electroporated with SV40 large T antigen. Only feeder cells without spermatogonia can be observed.* **(B,C)** *Clone of electroporated spermatogonia with their typical growth, forming colonies (5 weeks post-electroporation). A and B are phase contrast pictures while C is an immunofluorescence image showing staining with an anti c-kit antibody conjugated to FITC. Magnifications: A, 120X; B, 300 X; C, 500X.*

Using this method to obtain a pure type A spermatogonia cell line could be very useful for many experimental approaches, like the study of *in vitro* spermatogenesis and the transplantation of genetically modified germ cells in seminiferous tubuli (Ogawa *et al.,* 1997 and Meachem *et al.,* 2001).

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