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A method to remove follicle cells of *Ciona intestinalis* eggs

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Proteinase K was successfully used to remove follicle cells from the eggs of the ascidian ***Ciona intestinalis*** *prior to fertilization. This follicle cell removal facilitated the observance of 4,6-diamidino-2-phenyl-1-indol (DAPI) staining for DNA throughout development from fertilized egg to tadpole.*

Key words: *Ascidians, Ciona intestinalis, DAPI staining, 4,6-diamidino-2-phenyl-1-indol, follicle cells, proteinase K*

INTRODUCTION

Ascidians are model systems for studying various aspects of development. These aspects range from localizing specific macromolecules within a species to elucidating mechanisms of change resulting in new species (Jeffery and Swalla, 1992). Thus, the physiological and evolutionary perspectives of development can be studied. It is important to be able to detect developing tissues,cells, cell organelles, and macromolecules at all stages of development for comparisons within and among species. This is often difficult in ascidian eggs and embryos because of the presence of a complex follicle which persists until hatching. The follicle consists of an outer layer of follicle cells, an acellular chorion, and an inner layer of test cells. These cells are often opaque and obscure the events that occur during embryonic development.

The work presented here describes a proteinase K treatment protocol that removes the follicle cells of the *Ciona intestinalis* egg. *Ciona intestinalis* was the species chosen for study due to local availability and ease in obtaining gametes. In addition, *Ciona intestinalis* has one of the most complex ascidian follicles (Fig 1). The procedure described in this paper facilitates observation of cells and cell organelles, but does not interfere with normal development.

METHODS

Obtaining eggs and sperm

Gametes were obtained from large, gravid animals, collected at Coquimbo, Chile. The tunic and body wall were sliced to expose the sperm duct and oviduct. Gametes were obtained from each duct and kept separately until insemination.

Removal of follicle cells

Eggs were treated with 5 ml of a solution containing 0.5 μg/ml proteinase K in sea water for 30 min at room temperature. After this incubation, the eggs were observed by microscopy and compared to untreated controls. To remove the proteinase K, the eggs were centrifuged in a manual bench top centrifuge and re suspended in sea water containing 10% bovine serum albumin.

Insemination and development

Sperm was added to eggs in 10 ml sea water. Sperm and eggs from different individuals were used to overcome barriers to self-fertilization. Embryos were allowed to develop at 18° C. At this temperature, gastrulation began approximately 4 h after insemination.

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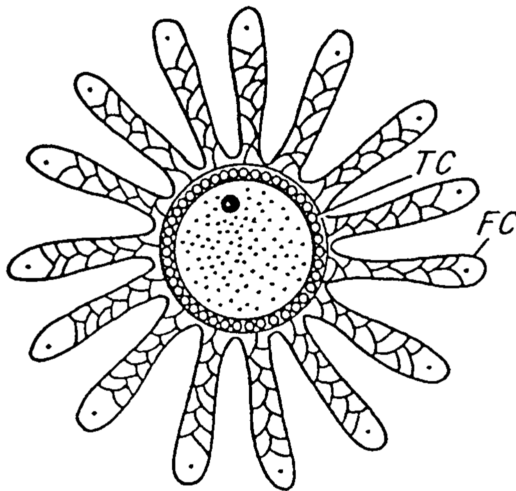

Fig 1. Follicle of *Ciona intestinalis*. FC, follicle cell; TC, test cell. The sphere within the egg is the nucleus.

TABLE I

Time table for *Ciona intestinalis* development (18° C). From Costello and Henley (1971)

Stage	Time after fertilization
2 cell	60 min
4 cell	90 min
8 cell	120 min
16 cell	160 min
32 cell	210 min
64 cell	250 min
Early gastrula	5 h
Neurula	7 h
Tadpole	18 h

Fixation

Embryos were fixed in 1 part 95% ethanol: 1 part sea water at 4° C at 210 min (32 cell), 250 min (64 cell), 5 h (early gastrula), and 18 h (tadpole) after fertilization (see Table I). The control was fixed at 18 h after insemination. Fixed embryos were stored at 4° C.

Staining

The fixed embryos were collected by centrifugation in a clinical centrifuge (setting 5), the supernatant was removed, and the pellet was resuspended in phosphate-buffered saline (PBS). This step was then repeated but the embryos were resuspended in 10 ml of 0.01% Na azide/PBS. After another cycle of centrifugation, 4,6-di-amidino-2-phenyl-1-indol (DAPI) stock solution was added for a final concentration of 5 μg/ml. Staining was carried for 30 min in the dark.

RESULTS AND DISCUSSION

Treatment of unfertilized *Ciona intestinalis* eggs with proteinase K was effective in removing the follicle cells. This was accomplished without deleterious effects, permitting normal development after fertilization. Fertilization was achieved subsequent to proteinase K treatment. Development of the embryos was observed through the tadpole stage for the proteinase K treated as well as the control zygotes. DAPI was used to determine the development of the embryos. The DAPI stained well, allowing observation of the location and number of nuclei in the embryos.

The use of proteinase K treatment for the removal of follicle cells prior to fertilization may provide an important method for examination of development in ascidian embryos. Follicle cell removal increases the ease of observation with DAPI stained embryos, and fertilization can still be achieved after follicle cell removal. This method will allow further research in this area. For example, it will now be possible to determine the localization and/or movement of actin filaments in the developing ascidian embryo without hindrance by the follicle cells.

Proteinase K treatment may prove useful in the removal of follicle ells in a variety of ascidian species. Due to the fact that *Ciona intestinalis* eggs can be fertilized with the chorion on or off, the present study does not provide evidence on whether or not the chorion was removed by proteinase K treatment. This information is of importance when considering the use of proteinase K treatment, because some ascidian species can only be fertilized with the chorion on the egg (Jeffery, 1992).

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