The Establishment of A Temporary Expression System in Chicken Oviduct Epithelium[1]

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ABSTRACT In the development of techniques by using a bioreactor to produce a special protein, and the establishment of a temporary expression system from primary cell culture of special tissue to analyze the expression regulators and the recombinant gene will greatly expedite the progress of research. This paper is the first report on the establishment of a temporary expression system from the primary culture of chicken oviduct epithelium, which continuously synthesized and secreted ovalbumin (OA) during more than two weeks of incubation. When the secretion function of the cells decreased, it could be restored in most of the cells by adding hormones in the culture medium within one week of incubation. The OA can be quickly and easily measured by using dotimmuofiltration assay in the cell culture medium because it is a kind of secretary protein. In order to examine whether the exogenous genes can be expressed in the primary culture cells, the green fluorescent protein gene (GFPG) was transfected into oviduct cells, and green fluorescence was observed in the cytoplasm of the epithelial cells. These results indicate that the temporary expression system in chicken oviduct epithelium is an effective and convenient system to analyze the expression regulators and the recombinant gene.

Keywords: chicken oviduct epithelium, temporary expression, bioreactor

1. INTRODUCTION

The success in the technology of transgenic animal inspired the idea of using an integral animal or plant as a bio-expression system to express the desired protein product encoded by a specific target gene. In an integral animal or plant the expression of desired protein products must be subject to the physiological condition of the animal or plant used, and the variation of biological individuals complicates the research for finding the general regularity of a bio-expression system. Therefore, to develop a method by using a bioreactor to produce the special protein and to establish a temporary expression system of primary cell culture of specific tissue to analyze the expression regulators and the recombinant gene will greatly expedite the research.

In China, there have been many reports on the development of mammary gland bioreactors. Another possible attractive approach is to produce foreign proteins in hen eggs because of the relatively short

generation time of chicken and the large number of progeny. So far, no research using oviduct as a bio-expression system has been reported. In one of our ongoing projects, a desired target gene was fused with the control element of ovalbumin (OA) gene as the recombinant gene and introduced into a fertilized chicken egg. Through the regulation of OA gene and the expression of ovalbumin in oviduct cells, the desired protein of the target gene would be produced in the egg. A temporary expression system in cellular level will facilitate the research on the structure and function of the recombinant gene. Therefore it is important to establish a temporary expression system in the primary culture of oviduct epithelium.

2. MATERIALS AND METHODS

2.1. Primary culture of chicken oviduct epithelium[1~3]

2.1.1. Separate cell cultures

Purebred laying hen provided by the Chinese Academy of Agriculture, Institute of Domestic Animals, was shaved at the abdomen and the neck was cut to shed blood. The abdomen of the hen was disinfected and dissected. The left oviduct was removed and a piece of 3~5 cm was taken in the magnum portion and placed into PBS with antibiotics. The mucous was separated from the magnum portion fragment under aseptic condition and the tissue fragment was washed once or twice with PBS containing antibiotics. The tissue was minced into very small pieces and digested at 37℃ in 0.25% trypsin dissociation solution (Trypsin, Amersham Life Science) by routine method. The tissue residue was removed by filtering the trypsinized tissue with layers of gauze. The separated cells was collected and washed once or twice with PBS containing 2% serum and the cells was resuspended in medium DMEM/F12 (Gibco) containing 2~5% fetal bovine serum (FBS Gibco). The cell suspension was further inoculated into a 6-well culture plate and were cultured at 37℃ with 5% CO_2. The medium was changed every day in the first week so as to remove unattached cells. In the following weeks, the medium was changed twice a week, and 0.1% bovine serum albumin (BSA) was used to replace FBS in the medium. In some experiments, a mixture of hormones such as 10^{-7} mol/L 17 β -estradiol (Sigma), 10^{-6}mol/L Corticosterone (Sigma) and 50 ng/mL Insulin (Sigma), was added to the medium as required.

2.1.2. Tissue mass cultures

Dissected tissue or residue of trypsinized tissue was cultured in 6-well plates. Complete medium was added when the tissue became slightly dry and changed twice a week.

2.2. Measurement of OA in the cell culture medium

Ovalbumin is a kind of secretary protein so one can measure it from the cell culture medium. To verify whether the primary culture of epithelium cells maintained the function of secreting OA, we

established the dot immuofiltration assay (DIFA) method to measure the OA in the cell culture medium. A fixed quantity of culture medium from oviduct cells, which had been cultured for different periods, was collected and concentrated onto a nitrocellulose membrane with negative pressure suction. A blocking solution (1%BSA, 0.25% Tween 20), rabbit anti-chicken-OA antibody (1:2 000, prepared by our lab) and horseradish super-oxidase conjugated protein A (1:5 000, Sigma) were added to the reaction system in sequence. Finally DAB (Zhongshang Bio Company, Beijing) was used to visualize the reaction. Positive reactions appeared as brown dots.

2.3. Liposome transfection of primary cultured cells

The green fluorescent protein gene (GFPG) has been used as a reporter gene. The advantage of using GFPG to study the function of the promoter is simple and the result can be observed directly. In order to examine whether the exogenous genes was expressed in the primary culture of chicken oviduct epithelium, the GFPG (pEGFP-N1, Clontech Laboratories, Inc.) was transfected into oviduct cells which had been cultured for 15 days. The transfection was mediated by DOTAP liposomal transfection reagent (Boenringer Mannheim). Transfection protocol: 1 μg of GFP DNA was dissolved in 100 μl of medium and 5 μl of DOTAP in 100 μl of medium. The DNA and DOTAP solutions were combined and incubated at room temperature for 20 min, and 800 μl of medium were added after that. For cells to be transfected, the old medium was changed with 1 ml of fresh serum-free medium in each well. DNA and liposome mixture was added to each well and incubated at 30℃ for 4 h. The medium was changed with the complete medium. The cells were examined 24 to 48 h after that.

3. RESULTS

3.1. Primary cultures of oviduct epithelium

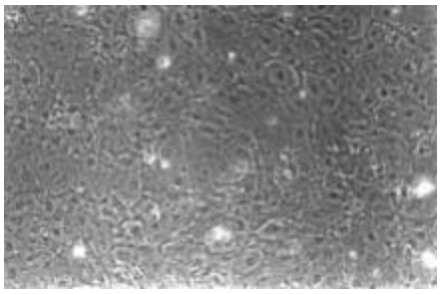

Fig.1 Primary culture of chicken oviduct epithelium, Day 10 (400 times).

During the first 5 days after inoculation, cell clusters attached to the bottom of the culture dishes and spread out. After about 10 days, cells formed a monolayes and colonies appeared, which showed the characteristics of epithelial cells (**Fig.1**). Secretion granules were observes in part of the cells. Later

vesicles appeared in some of the cells after approximately 4 weeks (**Fig.2**). In the continuous cultures no obvious proliferation of cells was observed, but the cells were maintained in fairly good condition.

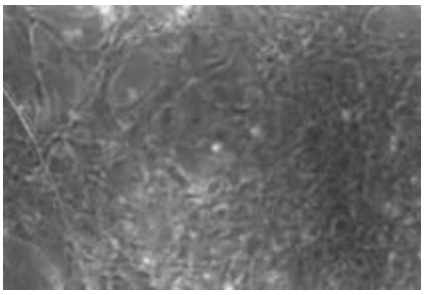

Fig.2 Vesicles were observed in primary culture of chicken oviduct epithelium, 4 weeks (400 times).

In tissue mass culture, out grown cells were observed only after day 10. The cells in the growth halo appeared in regular mosaic form with tubular gland cells arranged on the outside (**Fig.3**). The out grown cells ceased from expanding when they reached certain extent. The cells were able to be revived from a stock kept in liquid nitrogen and grew well.

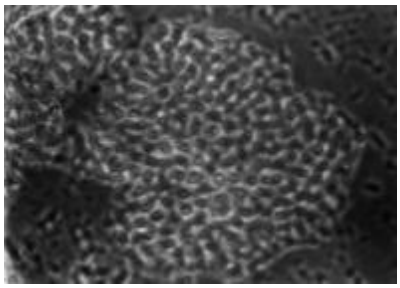

Fig.3 Typical epithelial growth halo in primary culture of tissue mass, Day 25 (400 times).

3.2. Results of OA measurement

In **Fig.4**, the left picture shows that the secretion of OA gradually decreased after the cells were cultured for a period of time, while the right picture shows the OA measurement of the same cells treated with hormones for one week. In both pictures the upper left dot was a positive control well with 1 μg of OA, and the lower right dot was a blank control well with BSA. The rest of the dots were samples. Cells continuously synthesized and secreted OA during more than two weeks of the incubation period. The secretion function of cells in the primary culture decreased within one month, but could be restored by adding hormones in the culture medium. The secretion of the cells failed to be restored by these hormones after more than one month.

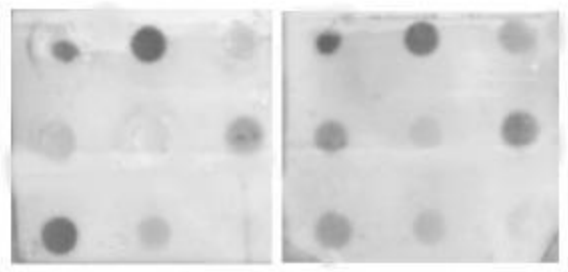

Fig. 4 The left picture shows that the secretion of OA gradually decreased after day 10 to 25, while in the right picture at the corresponding loci it shows the OA measurement of the same cells treated with hormones for one week. In both pictures, the upper left dot was an OA positive control well, and the lower right dot was a blank control well with BSA. The rest of the dots were for samples.

3.3. Liposome transfection of primary cultured cells

The cells were observed under the fluorescent microscope 24-h after the primary cultured cells were transfected by GFP gene. Green light could be observed in the cytoplasm of epithelial cells (**Fig.5**). These results demonstrate that the exogenous gene can be expressed in the primary culture of oviduct cells. In order to obtain the regulatory sequence which are necessary for generating the transgenic chick bioreactor for the specific expression of foreign gene in the oviduct, another researcher in our lab isolated a 2995 bp fragment at the 5' of chicken ovalbumin gene (OVP) by PCR. The construction of OVP with GFP was able to transiently express in the primary culture of oviduct epithelial cells. This result suggests that the regulatory gene OVP possess the specific transcription activity of oviduct.

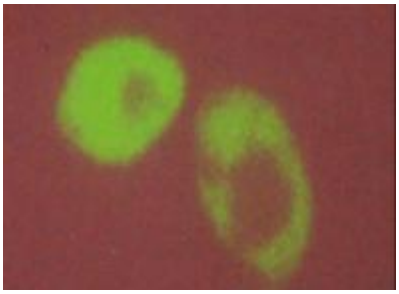

Fig.5 The GFP gene expression in oviduct cells. Fluorescent light was found in the plasma of cells (1 000 times).

4. DISCUSSION

In gene engineering industry it is a hot topic to apply gene-engineering technology to produce some protein products that are difficult to obtain by other methods. Previously, exogenous genes were expressed in prokaryotic cells, but in these cells there were problems in post-translation processing of desired proteins completely and correctly. Generally, proteins produced by mammalian cells possess

biological functions, but the culture of mammalian cells in large amount involves complicated technology and facilities. Recently, the target gene was introduced into an integral animal or plant, and desired proteins were expressed and produced accordingly. In order to express a target gene in specific tissues, regulating elements of a gene (such as OA gene) synthesized by these specific tissues should be used to control the target gene. Therefore a good understanding of the structure and function of the regulating elements of genes is the premise in preparing gene components. Using a temporary expression system to analyze the structure and function of regulating elements of genes has a unique role in this type of research, which a transgenic animal can not do. In this paper we report the methods and experience in establishing a temporary expression system in the primary culture of chicken oviduct epithelium.

4.1. The selection of digestive enzyme

The enzyme collagenase works very well in digesting the intercellular tissue and will separate the epithelium from collagen without damaging the epithelium. Therefore it is often used in the dissociation of epithelial tissues. In our experiments, collagenase had been used with good effect, but it is very costly and works only with the existence of other enzymes. Trypsin worked very well in our experiments too, so long as the duration of digestion was controlled well.

4.2. The supplementary ingredients in the medium

It is routine to use 5~10% serum to promote the growth of the cells. We tried to use a medium with 5% FBS. However, the over growth of fibroblasts inhibited the growth of epithelium. The problem was overcome by using a medium with only 2% FBS for 2~3 days, and replacing the FBS with 1% BSA after that.

4.3. The application of hormones

Estradiol, corticosterone and insulin are the major hormones that regulate the expression of OA gene. In the early stage of primary culture the effect of these hormones on cell growth and secretion of OA was not very obvious. However, when the cells were cultured for a period of time the secretion of OA in cells decreased or even stopped. These hormones would restore the secretion of OA in the cells.

4.4. The substrate in the medium

Generally, the culture of epithelium requires specific matrix in the medium, such as collagen, which may help the cell to attach, survive and proliferate. We tried to use mouse-tail collagen, chicken plasma, and extract of chicken embryo as substrate in the culture medium, respectively, but the results were not satisfactory. Meanwhile the use of these substrates made the culture of cells more laboursome. We have achieved good result without using any matrix in the medium.