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Mammalian nuclear transplantation to Germinal Vesicle stage *Xenopus* oocytes – A method for quantitative transcriptional reprogramming

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ABSTRACT

Full-grown *Xenopus* oocytes in first meiotic prophase contain an immensely enlarged nucleus, the Germinal Vesicle (GV), that can be injected with several hundred somatic cell nuclei. When the nuclei of mammalian somatic cells or cultured cell lines are injected into a GV, a wide range of genes that are not transcribed in the donor cells, including pluripotency genes, start to be transcriptionally activated, and synthesize primary transcripts continuously for several days. Because of the large size and abundance of *Xenopus laevis* oocytes, this experimental system offers an opportunity to understand the mechanisms by which somatic cell nuclei can be reprogrammed to transcribe genes characteristic of oocytes and early embryos. The use of mammalian nuclei ensures that there is no background of endogenous maternal transcripts of the kind that are induced. The induced gene transcription takes place in the absence of cell division or DNA synthesis and does not require protein synthesis.

Here we summarize new as well as established results that characterize this experimental system. In particular, we describe optimal conditions for transplanting somatic nuclei to oocytes and for the efficient activation of transcription by transplanted nuclei. We make a quantitative determination of transcript numbers for pluripotency and housekeeping genes, comparing cultured somatic cell nuclei with those of embryonic stem cells. Surprisingly we find that the transcriptional activation of somatic nuclei differs substantially from one donor cell-type to another and in respect of different pluripotency genes. We also determine the efficiency of an injected mRNA translation into protein.

1. Introduction

An amphibian oocyte in meiotic prophase is a remarkable cell. The single full-grown *Xenopus* oocyte is 1.2 mm in diameter. Its tetraploid nucleus is 400 μm in diameter and contains hugely expanded lampbrush chromosomes [1], which are much more active in transcription than the nucleus of any other cell-type [2]. During its approximately nine months of growth, it accumulates many different proteins and RNAs needed for development of the embryo. Some of these components such as histones are in sufficient abundance in the mature oocyte to provide chromatin for the 12,000 nuclei of an embryo before transcription of the zygotic genome starts [3]. Each oocyte contains 10^{12} ribosomes, enough for the formation of a tadpole consisting of 10^5 cells. Consequently, the anucleolate mutant, which has no ribosomal genes, survives till the feeding tadpole stage [4]. Therefore the *Xenopus* oocyte actively synthesizes huge reserves of components needed for development by prolonged and intense transcriptional activity.

Apart from its inherent interest, the oocyte is also extraordinarily useful as a cell to which macromolecules and complexes can be easily added. It is in effect a living test-tube, because a desired amount of any component can be introduced into its cytoplasm or its nucleus, and the injected oocyte can then be cultured in a salt solution for up to a month [5]. The value of *Xenopus* oocytes for injection first became evident when they were used to investigate the origin and identity of components required for DNA replication [6]. Since then, injected *Xenopus* oocytes have become increasingly useful for studies of protein regulation, RNA synthesis and the reprogramming of transplanted nuclei [7]. Here we review the characteristics of a *Xenopus* oocyte that enable it to switch the transcriptional program of transplanted somatic

Abbreviations: GV, Germinal Vesicle; MBS, Barth-Hepes saline; PBS-BSA, phosphate buffered saline containing bovine serum albumin; SLO, streptolysin O; DTT, dithiothreitol; SuNaSp, sucrose, sodium chloride, spermine and spermidine; SuNaSp-BSA, SuNaSp with bovine serum albumin; RA, retinoic acid; ES, embryonic stem cell; ESRA, embryonic stem cell, treated with RA to differentiate and cease Oct4, Nanog and Sox2 transcription.

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nuclei to that of an oocyte or embryo, a subject of much current interest in relation to the generation of embryo or stem cells from the differentiated cells of an adult.

Because one mature oocyte has the same volume as about 10^5 somatic cells, conclusions can be drawn about the behaviour and properties of this single defined cell-type. It is hard, and perhaps impossible, to obtain such large numbers of identical somatic cells because most adult tissues consist of very many different cell-types, in different stages of the cell cycle, and in different states of differentiation or function.

2. Results

2.1. Preparation of oocytes and permeabilized cells

Oocytes should be removed from a frog, as whole ovary tissue, after terminal anaesthesia by submersion in 500 ml of water containing 1 g of Tricaine Methane Sulphonate (MS222) or by subcutaneous injection of 120 mg (in 300 μl) of MS222. This is the most humane method of termination available, with pithing being wholly inappropriate for use with *Xenopus*.

When removed from a *Xenopus* ovary, each oocyte is surrounded by several thousand follicle and other cells (including blood vessels) and these make it very hard to penetrate the oocyte even with sharp injection needles. For many years, it was customary to peel off with forceps all except the innermost layer of follicle cells. Chemical defolliculation is much more convenient and faster, and in our experience is no more damaging to oocytes than mechanical defolliculation. This procedure is described by Astrand et al. [8]. Seven units of Liberase Blendzyme III or of Liberase TM Research Grade (Roche) (made up at 28 U/ml in H_2O) are added to 3 ml of, well rinsed, loosely packed oocytes, which have been torn into groups of 30 or less, in 12.5 ml of MBS [9] (as a total volume) in a 50 ml Falcon tube. These are incubated with gentle rocking for 2–2.5 h at room temperature until the follicle cells have been separated (Fig. 1). The defolliculated oocytes are extensively washed in MBS and maintained in MBS, at 14–18 °C, for several days until required.

Donor cells must have their plasma membrane permeabilized if their nuclei are to be exposed to the GV or cytoplasm of a recipient oocyte. Compared to digitonin, lysolecithin, and some other reagents, Streptolysin O (SLO) (Sigma–Aldrich, S5265) can conveniently permeabilize the plasma membrane but not the nuclear membrane [10]. SLO was shown to be harmless when tested by traditional nuclear transfer to eggs [11]. Trypsinized cells must be washed thoroughly to remove as much serum as possible before SLO treatment, since proteins can inhibit SLO activity. SLO stock solutions are prepared in PBS (containing 0.01% BSA and 5 mM DTT) at 20,000 units/ml, warmed at 37 °C for 60 min, and stored in 25 μl aliquots at −80 °C. The exact procedure varies according to cell-type and preparation of SLO, but a standard procedure is to add 20 μl (400 units) of stored SLO to 10^6 cells in 50 μl of SuNaSp [12], and incubate at 37 °C for 4 min. During or after this incubation, a few μl of cells can be added to 8 μl of 0.2% Trypan Blue in SuNaSp to determine the degree of permeabilization, seen as blue nuclei. We use preparations of cells that have been 95–99% permeabilized. As soon as permeabilization is sufficient, the reaction is stopped by adding an excess of SuNaSP BSA. A suspension of 10–25,000 permeabilized cells per μl in a suitable injection medium is used and this may be maintained on ice for a few hours until use.

2.2. Equipment for nuclear transfer to oocytes

The procedures and equipment for transplanting nuclei to oocytes have been described before [13,14]. Control of needle injection can be achieved by use of a Singer micromanipulator [9] and an Agla syringe [9], a Narishige (Narishige IM-300 microinjector, Narishige Scientific Instruments Lab., Japan), or Drummond (Drummond Nanoject, Drummond Scientific Company, USA) microinjector. We use a microscope stage equipped with temperature-controlled circulating water (usually at 15 °C) to keep the recipient oocytes cool during nuclear transplantation.

The needle profile is important for accurate GV targeting. A sharp glass needle (30–60 μm diameter tip) resembling that of a hypodermic needle is ideal. A number of different methods of achieving correctly shaped needle ends are available. The simplest of these involves crushing the tip until a sufficiently smooth but sharp edge is achieved. This type of needle can then be forged to give a sharper profile, or ground on a micro-grinder for a smooth and sharp finish (Fig. 2A).

The targeting of nuclei to the GV may be enhanced by marking the needle at roughly 250–300 μm from the tip with a fine laboratory marker to indicate the depth to which the needle should be inserted to deposit nuclei in the GV with a 80% or better success rate (Fig. 2B).

2.3. Injection into the Germinal Vesicle

The major challenge in GV transplantations is to correctly target the nuclei to the interior of the GV and not the oocyte cytoplasm. This is done by positioning the oocyte with forceps, such that the pole of the animal hemisphere is at an approximately 45° angle

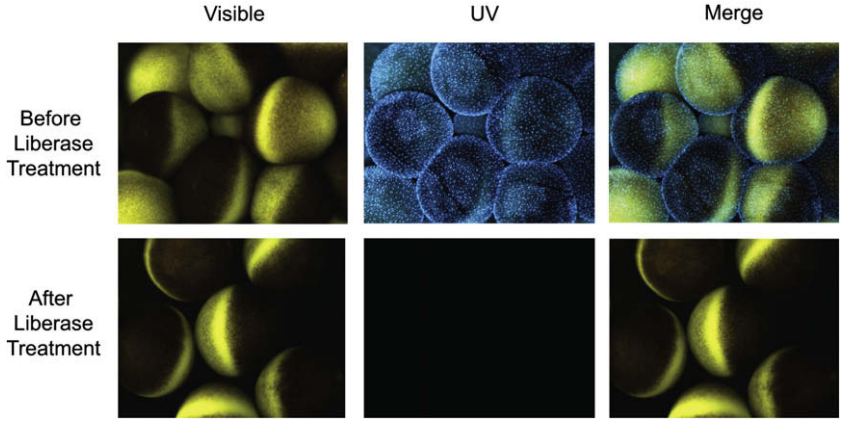

Fig. 1. Chemical defolliculation of oocytes completely removes the oocyte follicular cells, illustrated here by Hoechst (UV) staining of follicular cell nuclei, before and after Liberase treatment.