



The Egg and the Nucleus: A Battle for Supremacy

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BACKGROUND

As a brand new graduate student starting in October 1956, my supervisor Michail Fischberg, a lecturer in the Department of Zoology at Oxford, suggested that I should try to make somatic cell nuclear transplantation work in the South African frog *Xenopus laevis*. There were good reasons for wanting to do this (see below). The very important question to be addressed at that time was whether all cell types in the body have the same set of genes. This question had been asked by embryologists since 1886 (Raubert 1886), and Spemann (1938) had demonstrated by an egg ligation experiment that the nuclei of an eight-cell frog embryo are developmentally totipotent. It was clear that a definitive experiment required the replacement of a zygote nucleus by a somatic cell nucleus, asking whether the somatic nucleus could functionally replace the zygote nucleus by eliciting normal development of the enucleated recipient egg (Fig. 1). Briggs and King (1952) had already succeeded in transplanting a blastula cell nucleus into an enucleated egg and obtaining normal tadpoles in the frog *Rana pipiens*. However, Briggs and King (1957) had also found that the nucleus of an endoderm cell from a neurula embryo could no longer support normal development (Fig. 2). They drew the reasonable conclusion that, as development proceeds from a blastula to a neurula stage (about 24 hours), some genes needed for normal development had either been lost or irreversibly repressed.

To this extent, the aim of my proposed PhD work had already been done and the answer to the primary question already obtained. Why then did it make

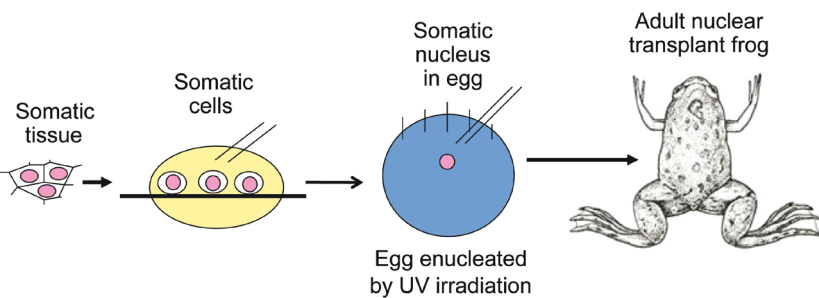


FIGURE 1. Design of a somatic cell nuclear transfer experiment using unfertilised eggs as first designed by Briggs and King (1952) for *Rana pipiens* and as used subsequently in *Xenopus*. In *Rana*, enucleation is by hand with a needle, and in *Xenopus* by ultraviolet light irradiation (Gurdon, 1960a).

sense for me to try to repeat this work on a related species? There appeared to be two possible outcomes. One was that I might obtain a different result from Briggs and King and so the primary question would be re-opened and subject to fruitful investigation. The other was that I might obtain the same result as Briggs and King and this would then open the important question of what the mechanism could be by which a somatic cell nucleus already committed to a specific (in this case endoderm) fate could not be reprogrammed by exposure to egg cytoplasm.

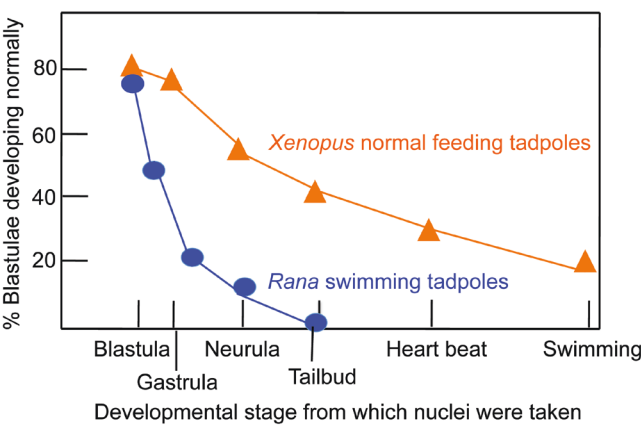


FIGURE 2. Survival of nuclear transplant embryos in *Rana pipiens* and *Xenopus laevis*. Even advanced donor cells from the endoderm have nuclei which can sometimes yield normal individuals after nuclear transfer (from Briggs and King 1957 [*Rana*] and Gurdon 1962 [*Xenopus*]).

Preliminary investigation showed that there would be substantial technical difficulties in achieving somatic cell nuclear transfer with *Xenopus*, in the way that Briggs and King succeeded for *Rana pipiens*. The first of these was that, unlike *Rana*, the *Xenopus* egg is covered with a dense and extremely elastic jelly, which is completely impenetrable by even the finest of micropipettes (Fig. 3). The second was that this jelly covering made it very hard, even if possible, to remove metaphase egg chromosomes by causing extrusion of them with a needle, the method used in *Rana pipiens*. There were, on the other hand, very good reasons to wish to make this technique succeed in *Xenopus*. How *Xenopus laevis*, a native of South Africa, came into use for Developmental Biology has an amusing and serendipitous history (Gurdon and Hopwood 2000). First, *Xenopus* would respond to the injection of commercially available mammalian hormone

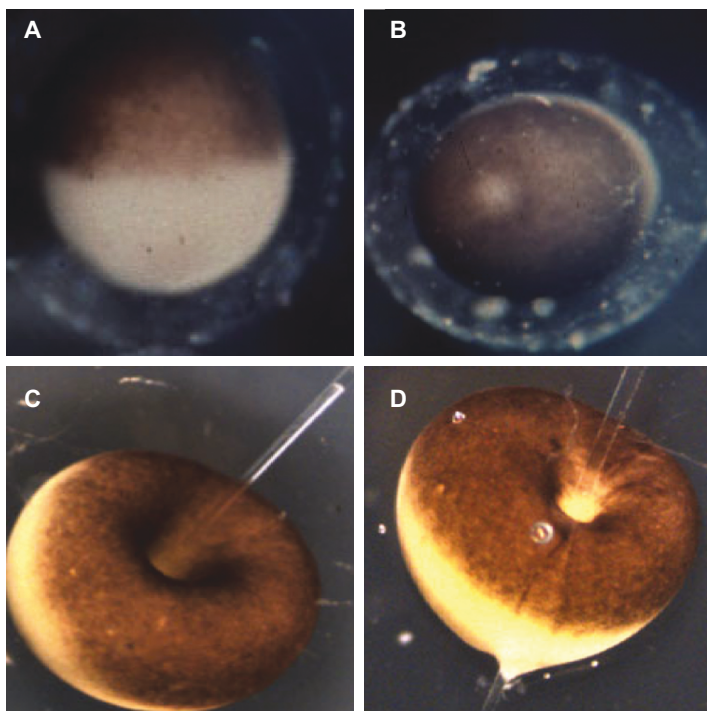


FIGURE 3. The *Xenopus* egg is surrounded by a dense elastic jelly so that it is not possible to penetrate into the egg cytoplasm with a micropipette, unless the jelly is removed or denatured by ultraviolet light. (a) side view. (b) “animal” pole; the white area in the middle of the black area is where the egg chromosomes are located. (c) If the egg is not de-jellied, a micropipette depresses the jelly coat, eventually dragging the pipette, still surrounded by jelly, through the egg without entering the cytoplasm (d).

(Follicle Stimulating Hormone and Luteinizing Hormone) by laying eggs the next day and this procedure is effective throughout the year. In contrast, frogs of the European and North American *Rana* species will lay eggs only in the spring of each year unless they are injected with frog pituitary gland extract, and this requires about five pituitary glands from killed frogs to obtain one egg ovulation. In the past, European embryologists had the use of living frog eggs for only a month or two in the year and had to do other things, such as histology *etc.*, for the rest of the year. *Xenopus*, in principle, permitted experiments on living embryos to be done throughout the year. Second, *Xenopus* is an aquatic frog and therefore easy to maintain in the laboratory in water tanks rather than having to clean a terrarium as was necessary with *Rana*. Furthermore, *Xenopus* species can be reared from the fertilised egg to sexual maturity in less than one year (compared to three to four years for *Rana*), thereby making it realistic to propagate genetic mutants and make use of them. A further advantage of *Xenopus laevis* is that this species lives in highly infected cattle sewage ponds and has built up an extraordinary resistance to infection and diseases. Michael Fischberg therefore concluded that it was sensible to have me try, at least for a while, to achieve successful somatic cell nuclear transfer in *Xenopus*.

The aim of this article is to recount the early history of nuclear transfer in *Xenopus* as a result of which the recent Nobel award was made (Jaenisch 2012). Further work in *Xenopus* that has led on from this up to the present time is covered only briefly, and has been reviewed elsewhere (Jullien *et al.* 2011; Pasque *et al.* 2011b).

THE TECHNIQUE OF NUCLEAR TRANSFER IN *XENOPUS*

There is no doubt that I was blessed with a considerable amount of luck. But the phrase that “luck favours the prepared mind” may well have been true. My supervisor had just acquired a microscope equipped with ultraviolet light illumination. There was reason to believe that ultraviolet light would destroy DNA in the egg chromosomes which, very fortunately, are located right on the surface of the animal pole of amphibian eggs. Aiming the ultraviolet light source onto the animal pole of unfertilised eggs was successful in destroying the egg chromosomes, as shown by fertilising such irradiated eggs with sperm and obtaining haploid embryos. If the egg chromosomes had not been located on the surface of a large amphibian egg, ultraviolet light (having very low penetration) would not have reached them (Gurdon 1960a). Perhaps even more fortunate was our finding that the particular ultraviolet lamp just bought for microscopy progressively denatured (dissolved) the elastic jelly around the egg. After ultraviolet

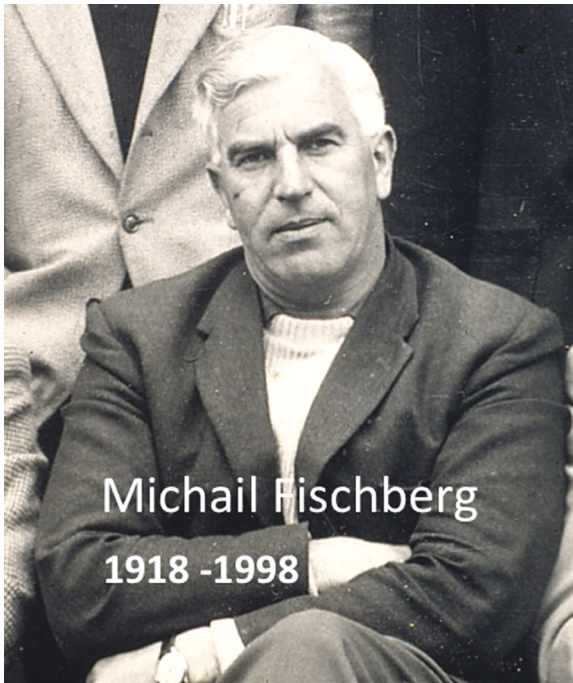


FIGURE 4. Michail Fishberg. Born in St Petersburg, educated in Switzerland and PhD under E. Hadorn. The education lineage traces back from Hadorn to Baltzer to Boveri. MF was my graduate supervisor in Oxford, England, from where he moved to Geneva.

light exposure, unfertilised eggs became easily penetrable by a micropipette, and this happened in a dose-dependent way, making it possible to enucleate the egg, leaving just enough jelly to help seal the penetration wound made by a micropipette. It was not known, at that time, that this egg jelly could be removed by an alkaline solution of cysteine hydrochloride, but good luck, or my supervisor's wisdom, or both, did not stop at this point. Crucial to the validity of these early experiments was proof that the egg chromosomes had in fact been destroyed and did not contribute to the development of the nuclear transplant embryos. Another PhD student of my supervisor, namely Sheila Smith, was studying the morphology of haploid development in *Xenopus*. She was advised to use a single nucleolus per nucleus as a measure of haploidy. She encountered an inexplicable result, namely that embryos carrying only one nucleolus per nucleus were diploid and developed entirely normally, whereas haploids (which have only one nucleolus per nucleus or chromosome set) always die as stunted early tadpoles. Most supervisors would have told the student to repeat the experiment the next week, starting with completely different material, to

see if the result was reproducible. Michail Fischberg (Fig. 4), however, had the wisdom or intuition to tell the student to find out which frog had been used to give the eggs that yielded normal diploid one-nucleolated embryos. Amazingly, the student’s result was reproducible. Michail Fischberg concluded that there must have been a mutation in one chromosome set so that it was unable to make a nucleolus (Elsdale *et al.* 1960). Later work showed that this strain of *Xenopus* had indeed lost all the ribosomal genes located in one nucleolus organizer and therefore that heterozygotes for this deletion would never carry more than one nucleolus per diploid chromosome set (Brown and Gurdon, 1964). This mutation gave us an extraordinarily valuable nuclear marker for nuclear transfer experiments (Fig. 5). Some years later, an albino strain of *Xenopus laevis* provided a more visually striking marker (Fig. 6).

Using the benefits of ultraviolet radiation, combined with a genetic marker, it was possible, rather rapidly, to show that somatic cell nuclear transfer in *Xenopus* worked well. Within one year of starting work, I had found that the nucleus of an endoderm cell from an advanced tadpole was able to yield some normal development up to the nuclear transplant tadpole stage. This was not in agreement with the results of Briggs and King (Fig. 2).

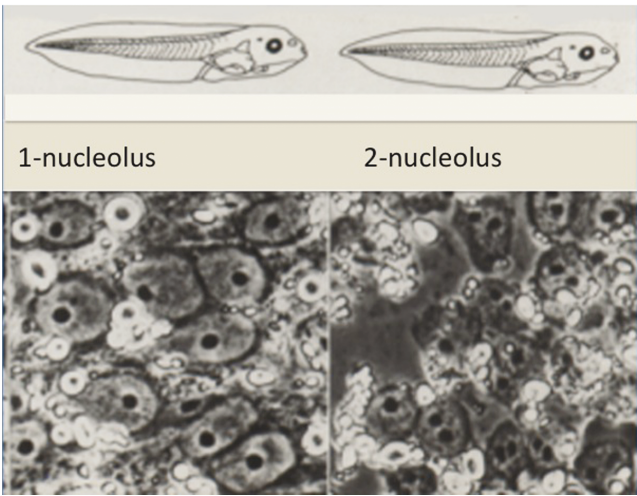


FIGURE 5. A nucleolar genetic marker for *Xenopus laevis* (Elsdale *et al.* 1960). Heterozygotes of the one-nucleolated strain have only one nucleolus per diploid nucleus (left), compared to the wild-type 2-nucleolated form most of whose nuclei have two nucleoli (right). The one-nucleolated strain has a deletion of ribosomal genes on one chromosome (Brown and Gurdon 1964).

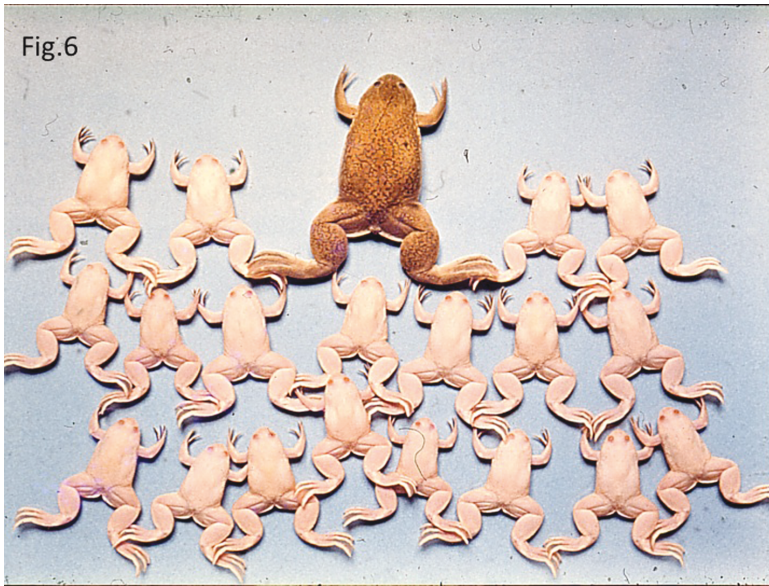


FIGURE 6. A clone of albino male frogs obtained by transplanting nuclei from cells of an albino embryo to enucleated eggs of the wild-type female shown. The albino frogs are genetically identical and will accept skin grafts from each other.

NORMAL DEVELOPMENT FROM THE NUCLEI OF DIFFERENTIATED INTESTINAL EPITHELIUM CELLS

Within another year, now 1958, I found that it was technically possible to transplant single nuclei from the intestinal epithelium of feeding tadpoles. I found it best to distort the donor cells to the least amount possible (Gurdon, 1960b), so that at least some of them had the nucleus in a ruptured cell wall, even though other such donor nuclei may have been transplanted in whole non-permeabilised cells, which however would not be able to respond to the egg cytoplasm or begin to cleave. It seemed important not to expose the nucleus of a ruptured cell to the simple saline medium used for nuclear transfer. It was later found that treating small donor cells with Streptolysin O was a great deal easier than cell rupture in a narrow pipette (Chan and Gurdon 1996).

The success of these intestinal epithelium nuclear transfers differed from one experiment to another. Moreover, some females supplying recipient eggs gave significantly better development than others. Egg quality was therefore a factor. Nevertheless, these results showed that starting with nuclei from differentiated intestinal epithelium cells, with a striated border, some of the nuclear transplant

embryos developed entirely normally to the feeding tadpole stage and were progressing towards metamorphosis. Furthermore, these tadpoles carried only one nucleolus per nucleus with a diploid set of chromosomes. This showed that the transplanted nucleus that gave normal development did indeed derive from an intestinal epithelium cell. Although the percentage of intestinal epithelium cell nuclear transfers that yielded entirely normal feeding tadpoles was low (1.5%) (Gurdon 1962) many such individuals were obtained and they all carried the nuclear marker.

My supervisor and his assistant looked after my nuclear transplant tadpoles, which had by now metamorphosed into young frogs, during my absence for postdoctoral work in another field. On my return, these intestinal nuclear transplant tadpoles had become adult male and female frogs, and their fertility and ability to generate normal embryos was tested. This yielded, in 1966, our paper entitled, "Fertile" intestine nuclei (Gurdon and Uehlinger 1966). This therefore gave the opposite conclusion to that of the Briggs and King work with *Rana pipiens*. Of course, there was criticism that a graduate student, working almost alone, should not be able to repeat the results of well-established and highly-respected workers Briggs and King. The use of the one-nucleolated genetic marker was crucial in persuading scientists that this *Xenopus* work was valid. In the course of time, it became accepted in scientific circles that cells can undergo complete differentiation, to the point of making intestinal epithelium cells of a feeding tadpole, without any loss or stable inactivation, of genes needed for entirely unrelated cell lineages and indeed for every cell type.

After these early experiments, the main conclusion that, during the course of cell differentiation the genome is conserved and repressed quiescent genes can be reactivated, was confirmed. With various colleagues, and especially R. A. Laskey, we were able to obtain normal tadpoles from adult foot web skin and from a range of adult organs such as heart, lung, etc. from cells grown out in culture from these tissues (Laskey and Gurdon 1970). Although we were able to obtain normal sexually mature male and female adult animals from the intestinal epithelium cells of a feeding tadpole and we were able to obtain feeding tadpoles from the nuclei of adult cells, we never obtained a sexually mature adult animal starting from the nucleus of another adult cell. We think that the intensely rapid cell division and DNA replication enforced on an Amphibian transplanted nucleus by an activated egg has a high probability of introducing replication defects, as is seen in *Rana pipiens* (Di Berardino and King 1967) thereby greatly reducing the chance of obtaining entirely normal development from the nucleus of an adult cell.

EPIGENETIC MEMORY

In addition to the rapid DNA replication and cell division enforced on a transplanted somatic nucleus, there are other ways in which we may account for the progressively decreasing success rate of nuclear transfers from differentiating and differentiated cells. One of these is that there may be a memory of a pattern of gene expression characteristic of the differentiated state. One obvious possibility is that there could be a resistance to the reactivation of those genes which are needed for early development but which have become quiescent or repressed during cell differentiation. This possibility is discussed below under the heading of "Resistance."

Another interesting possibility is that there could be a memory of an active gene state. For example, those genes that are strongly expressed in specialised cells might fail to be switched off after nuclear transfer and might then interfere with new directions of lineage selection by nuclear transplant embryos. Methods that were not available when amphibian nuclear transfers were first carried out have now made it possible to test this idea. Nuclei were transplanted from muscle or other lineage-specific progenitor cells to make nuclear transplant embryos. Although many of the resulting nuclear transplant embryos developed abnormally, it was possible to collect enough material from partially cleaved embryos to carry out gene-specific transcript assays. The surprising result was obtained that a considerable memory of an active gene state persisted in these nuclear transplant embryos through many cell cycles of inactive transcription characteristic of early amphibian embryos. We found that the neurectoderm and endoderm lineages of nuclear transplant embryos derived from muscle progenitor nuclei often continued to express muscle genes to an excessive extent (Ng and Gurdon 2005). The memory was imperfect, in that about half of the nuclear transplant embryos from muscle progenitor cells showed an excessive, sometimes very large, overexpression of muscle genes in inappropriate tissues whereas the other half did not (Fig. 7). Genes characteristically expressed in a certain lineage became repressed for the early cleavage divisions of a nuclear transplant embryo but then became re-expressed throughout the embryo after the stage of transcriptional activation at the late blastula stage. This result was also found in other, non-muscle lineages (Ng and Gurdon, 2005). It was also found that this "memory" of an active gene state was associated with histone H3.3, an abundant protein in eggs and early embryos. The explanation offered for this phenomenon was that the very high level of histone normally present in oocytes and eggs (Cho and Wolffe 1994) enhances transcription of any gene

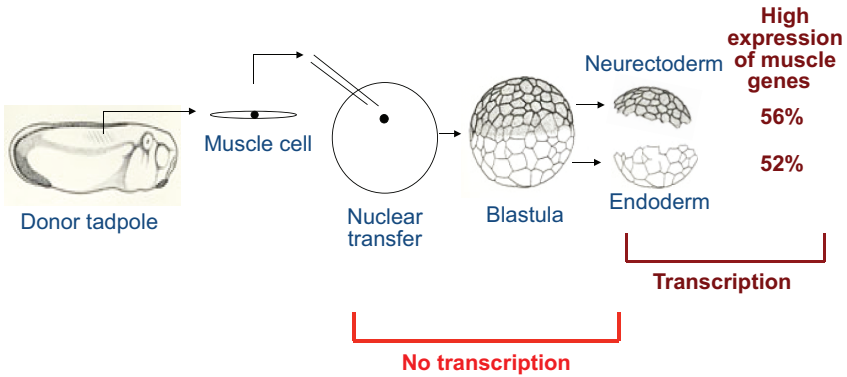


FIGURE 7. Epigenetic memory in nuclear transplant embryos. Nuclear transplant embryos derived from muscle nuclei were grown to the blastula stage, and then depleted of the mesoderm region (muscle lineage). The remaining regions (neur ectoderm for nerve/skin cells and endoderm for intestine lineages) express the muscle gene marker *MyoD* to an excessive extent in about half of all such embryos (Ng and Gurdon 2008).

that is in an active state at the time of nuclear transplantation (Ng and Gurdon 2008). Histone H3.3 is known to be associated with active transcription. Memory of an active gene state was subsequently described in iPS experiments (Polo *et al.* 2010). The observation that about 50% of nuclear transplant embryos show this memory that is not seen in the other 50% exemplifies the concept (see later) that there is a conflict between components of an egg that are designed to restore gene expression to that characteristic of an egg and embryo and the resistance of the nucleus of determined or specialised cells to resist any change, thereby stabilising the pathway of differentiation on which an embryonic cell has set out.

NUCLEAR TRANSFER IN MAMMALS

For these early results in *Xenopus* to be reproduced in mammals took nearly 40 years (Campbell *et al.* 1996; Wilmut *et al.* 1997) in sheep. A very important feature of these first successful mammalian nuclear transfer in sheep was the use of unfertilised eggs, as was actually used in amphibia. Earlier work with mice (McGrath and Solter 1984) used fertilised eggs. Although fertilised eggs can be used (Egli *et al.* 2007), synchronisation between nucleus and egg is harder to achieve than with the use of unfertilised eggs. A very elegant and important experiment that confirmed the general principle that cell differentiation proceeds

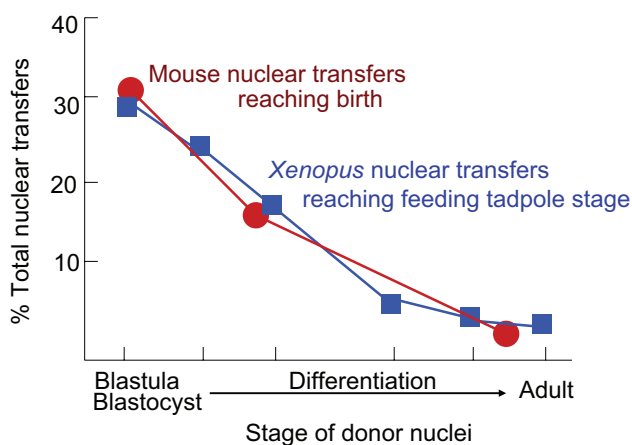


FIGURE 8. Survival of nuclear transplant embryos in *Xenopus* (Gurdon 1962) and the mouse (Wakayama *et al.* 1998).

with the retention of a complete set of genes was carried out using nuclei with a rearranged genome from mature mouse B or T donor cells (Hochedlinger and Jaenisch 2002). In the course of time, somatic cell nuclear transfer to eggs has been successful in the eggs of mice and other mammals (Wakayama *et al.* 1998). In each species there seem to be some technical requirements which have to be identified and overcome. In mammals, the early cell divisions after fertilisation are extremely slow compared to amphibians (20 hours from fertilisation to two-cell stage in the mouse). It is therefore unlikely that the chromosome damage seen in amphibian work (above) is important in mammals. Nevertheless the decreasing success rate of nuclear transplant embryo development is about the same in mice and frogs (Fig. 8). There must be other reasons for this resistance to reprogramming by eggs.

This brief history of successful somatic cell nuclear transplantation does not do justice to the many important contributions made after the early *Xenopus* work. For reviews of the early *Xenopus* work see Gurdon 1986. Subsequent reviews which also cover the early work have been published by McKinnell (1978), Di Berardino and Hoffner (1983) and Gurdon (2006).

MECHANISMS OF NUCLEAR REPROGRAMMING BY EGGS

The second question raised when embarking on a PhD thesis on nuclear transfer in *Xenopus* concerned mechanisms of reprogramming. There are two parts

to this question. First, how does an egg reverse the differentiation state of a somatic nucleus to enable it to behave like a zygote nucleus when it leads to entirely normal development? The second question asks in what way do the nuclei of somatic cells become progressively resistant to the reprogramming conditions of an egg?

To approach these questions it was clearly necessary to focus on the transcription of individual kinds of genes. At that time, in the 1960s, genes had not been cloned and it was only possible to work with genes which were present in multiple copies per genome, such as 28S, 18S and 5S ribosomal genes. Working with ribosomal genes, transfer RNA genes, and the gross class of genes whose base composition resembled the average of the genome, it was shown that transplanted somatic nuclei in blastula and gastrula stages of nuclear transplant embryos had reverted to an embryonic pattern of transcription (Woodland and Gurdon 1969). However this did not lead to understanding the mechanism by which this rejuvenation took place. It took a few decades before single genes expressed in early *Xenopus* development had been cloned and the necessary probes and procedures developed by which the expression of these genes could be monitored in nuclear transplant embryos.

At this time it seemed useful to investigate the mechanisms that guide early embryo development when nuclear transplant embryos, especially from advanced donor stages, often develop very abnormally. I wondered whether the nuclear transfer techniques could be used to introduce purified macromolecules into an egg, and hence into embryonic cells. Thanks to my scientific friendship with Jean Brachet of Belgium, a major contributor to the field of Developmental Biology (Brachet 1957), I was able to acquire a small sample of purified globin mRNA from the laboratory of Dr Chantrenne. He was one of the first to purify any animal mRNA. Even a few micrograms of this was more precious than gold dust, and anything that came in contact with it had to be chromic acid cleaned for fear of RNase. A highlight of my career at this time was the discovery that purified messenger RNA could be extremely efficiently translated into protein when injected directly into an egg or embryonic cell (Gurdon *et al.* 1971). Interestingly, this finding was completely unexpected because of the very high ribonuclease activity present in eggs. For this reason a grant application to permit such an experiment would not have succeeded. I was fortunate to have enough other funding to do this work without specific grant support. Amazingly it was possible to inject rabbit globin mRNA into the fertilised *Xenopus* egg, grow that egg to a tadpole stage and demonstrate that tissues such as muscle were still translating high levels of globin, wholly

inappropriate to that cell type, without any interference in normal development (Woodland and Gurdon 1974). The injection of messenger RNA, and other macromolecules, into an egg has now become a very widely used procedure in developmental biology. I am still amazed at how well this works. We can now understand that the injection of an egg with a micropipette is sufficiently harmless that ribonuclease is not released from egg cytoplasm. It may be that the penetration of an egg with a micropipette is no more harmful than the penetration of an egg by sperm after fertilisation. mRNA injection led to the widespread use of mRNA injection for over- and under-expression experiments in developmental biology.

A key mechanism in early development is the concentration-dependent response of cells to signalling molecules, an area known as morphogen gradient interpretation. Even two-fold differences in ligand concentration are enough to make competent embryonic cells choose which cell type lineage to follow (Green and Smith 1990; Dyson and Gurdon 1998). We now know that small quantitative deficiencies in signalling can adversely affect nuclear transplant embryo development, as shown in cross species nuclear transplantation (Narbonne *et al.* 2011). Another particularly interesting aspect of concentration-dependent signalling is illustrated by the community effect (Gurdon 1988). Resulting from single cell transplantations, the concept developed by which a group of similar cells can contribute a high enough concentration of signal molecule to exceed a threshold never produced by a single cell. This community effect seems to contribute to the normal development of multicellular tissues in development. It later turned out that the principle behind the community effect had already been proposed, as “quorum sensing,” to be involved in bacteria-dependent light emission in predatory fish and in other examples (Lamb 2012).

To progress with the analysis of reprogramming by egg cytoplasm, an obviously desirable route would be to achieve successful reprogramming of somatic nuclei by extracts of eggs; this could lead to the identification of such components by fractionation and selective depletion. Somatic nuclei transplanted to eggs are almost immediately induced to commence DNA replication (Graham *et al.* 1966). Extracts of eggs are remarkably successful in inducing DNA replication in isolated nuclei (Laskey *et al.* 1989; Mechali 2010) but such extracts are notoriously difficult to make in such a way that transcription proceeds meaningfully. This difficulty in making functional extracts of cells is in marked contrast to the long lasting success of injecting messenger RNA, genes, *etc.* into living cells. The injection of components into eggs and early embryo cells can be regarded as “living biochemical test tubes” (Gurdon 1974).

THE ANALYSIS OF NUCLEAR REPROGRAMMING BY OOCYTES

It was evident from the earliest amphibian nuclear transfer experiments that the replication of DNA and chromosomes in somatic nuclei transplanted to eggs is very often defective (Di Berardino and King 1967). Once penetrated and activated by sperm or an injection pipette, amphibian eggs immediately enter a phase of some ten or more rapid cell division cycles. It is very difficult for a somatic cell nucleus, which might normally divide once every two days, to switch immediately to a division cycle of 30 minutes. As a result, the DNA of transplanted nuclei or their daughters is often torn apart at cell division when incompletely replicated. This leads to major chromosome loss and other defects, especially when the nucleus of a slow-dividing somatic cell is transplanted. It was clear, at this point, that we had to find a way of analysing the reprogramming of somatic cell nuclei without the disadvantage and damaging effect of enforced rapid DNA replication and cell division. This led to the introduction of amphibian oocytes as somatic cell nuclear transfer recipients.

The amphibian oocyte is the growth phase of an early germ cell to a full-sized egg progenitor with lampbrush chromosomes over a period of several months (Callan 1982). These cells are in the prophase of first meiosis (Fig. 9).

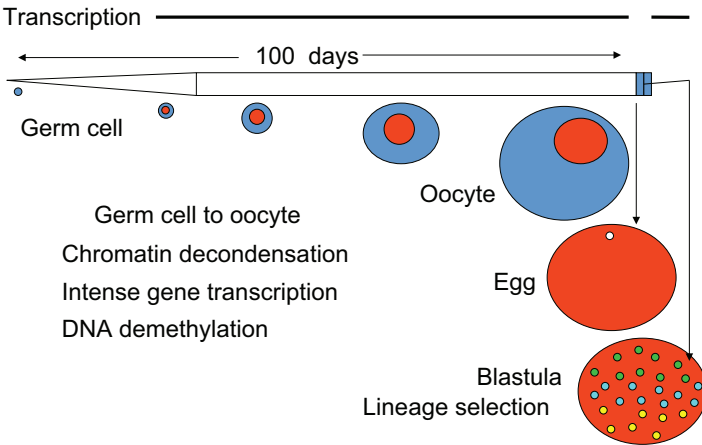


FIGURE 9. The *Xenopus* oocyte grows in the ovary from a germ cell over many months while it is in first meiotic prophase. When fully grown it can respond to hormones, such as progesterone, to complete first meiosis and arrest in second meiotic metaphase. Once fertilised, it progresses to the blastula stage in 8 hours and somatic lineages already start to appear.

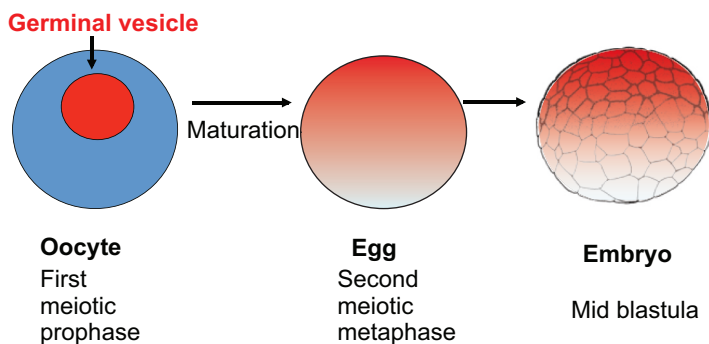


FIGURE 10. A *Xenopus* oocyte has a huge (420μ diameter) germinal vesicle, which includes its tetraploid chromosome set. After completion of meiosis, the germinal vesicle contents are distributed to the egg and subsequently to the embryo.

These full-sized egg progenitors are normally induced by hormone levels to complete first meiosis and progress to the metaphase of second meiosis, after which they can respond to fertilisation. While still in my PhD graduate work, I developed the use of *Xenopus* oocytes to analyse the origin of the DNA replication inducing capacity of eggs. Even sperm nuclei can be converted to lampbrush chromosomes after injection to oocytes (Gall and Murphy 1988). It became clear that somatic nuclei or even pure DNA would be efficiently and correctly transcribed when injected into the germinal vesicle (= nucleus) of an oocyte (Mertz and Gurdon 1977; Brown and Gurdon 1977). It is important to appreciate that the germinal vesicle of an amphibian oocyte contains an enormous reserve of developmentally essential components, which are distributed to the egg cytoplasm during completion of meiosis (Fig. 10). These reserves are necessary for normal embryonic development. Fortunately for developmental biologists, these components, specified by the intensely active lampbrush chromosomes, are accumulated in the specialised germinal vesicle where they represent a high concentration of components that later enter the egg cytoplasm. Since DNA replication and cell division do not take place in these growing oocytes, the oocyte germinal vesicle provides an accessible accumulation of transcriptionally-active components.

Somatic nuclei, chromatin, or DNA, of amphibian or mammalian origin, can, with practice, be injected into the invisible germinal vesicle of an intact oocyte (Halley-Stott 2010). *Xenopus* oocytes show selective transcription of somatic nuclei from unrelated species (De Robertis and Gurdon 1977). Transcription

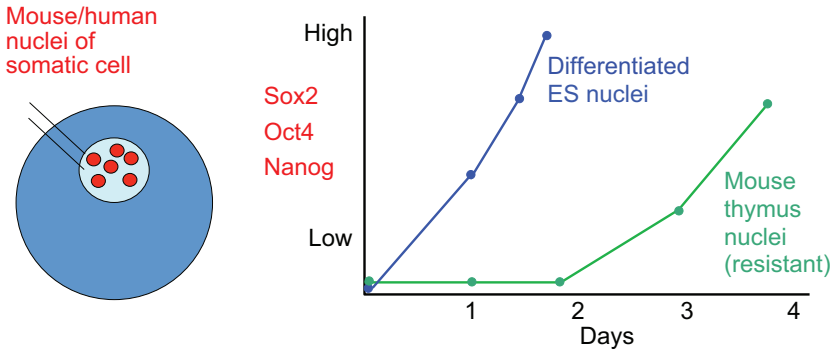


FIGURE 11. Somatic nuclei injected into the germinal vesicle of an oocyte transcribe genes that are quiescent in the donor cells but are rapidly transcriptionally activated. The most specialised donor nuclei (mouse thymus) showed temporal resistance to transcriptional activation.

of injected nuclei or genes takes place at a high rate, with as much as several hundred re-initiations of transcription on a gene per day. Two hundred to 300 somatic nuclei can be injected into one oocyte's germinal vesicle, so that one injected oocyte provides the same amount of nuclear material as 250 eggs injected with a single nucleus (Fig. 12). This makes it realistic to carry out on oocytes those molecular techniques that normally require large amounts of material. When injecting purified DNA, this becomes chromatinised (Wyllie *et al.* 1977). Oocytes continue to transcribe injected nuclei or chromatin for several days. It is possible to manually isolate the germinal vesicle from an oocyte, containing injected somatic nuclei, and to carry out antibody binding, FRAP assays etc. on individual transplanted nuclei. After injection into the germinal vesicle, somatic nuclei undergo a massive chromatin decondensation as does sperm in an egg. After transfer to oocytes, some genes are transcribed extensively, and continue to accumulate large numbers of transcripts. These activated genes include some of those that are active in embryos, including the well-known pluripotency genes, such as Oct4, Sox2, Nanog, etc. These characteristics make the injected first meiotic prophase oocyte of *Xenopus* very suitable for analysing both the activation of genes during reprogramming as well as the basis of resistance by the nuclei of differentiated somatic cells (Halley-Stott *et al.* 2010).

TRANSCRIPTIONAL ACTIVATION

Several necessary early steps have now been identified. The first of these is the movement of a special linker histone, known as B4 in amphibia or H1foo

in mammals, into transplanted nuclei. This histone protein is very abundant in the germinal vesicle of amphibian oocytes and a large amount of it is incorporated into the chromatin of injected nuclei within 2–3 hours at 17°C. This step is necessary for subsequent transcriptional activation, as shown by the use of antibodies and overexpressed dominant negative forms of this histone which inhibit subsequent pluripotency gene activation (Jullien *et al.* 2010). When B4 histone invades transplanted nuclei, these nuclei lose the somatic form of linker histone. This substitution of linker histone in chromatin is likely to be an important part of the striking decondensation of chromosomes that takes place soon after nuclear injection. This early event is thought to give access of other oocyte components, including transcription factors, to injected chromatin. B4 histone is abundant in oocytes but is not present in normal development after the blastula stage (Smith *et al.* 1988). The next important events include the movement of another oocyte-specific histone, namely histone H3.3, into injected nuclei. Histone H3.3 is present in somatic cells but at a much higher concentration in oocytes, and is generally associated with active transcription. We have noted above that histone H3.3 may be causally associated with epigenetic memory in somatic nuclei transplanted to second metaphase eggs. If histone H3.3 has a general role of enhancing transcription, this would help to account both for epigenetic memory in nuclear transplant embryos as well as for the increasing level of transcription seen in somatic nuclei transplanted to oocytes (Gurdon 1986). A later event is the polymerisation of nuclear actin in oocytes and in nuclei transplanted to their germinal vesicles (Miyamoto *et al.* 2011). This seems to enhance the level of transcription of transplanted nuclei during the first two days. This sequence of events leads to a high level of transcriptional reprogramming, and takes place at a remarkably fast rate. Within two days, most of the somatic nuclei transplanted to oocytes have strongly activated transcription of the pluripotency gene *Sox2*; this happens at 17°C, the metabolic equivalent of 12 hours at 37°C.

Although the transcription of some genes, after nuclear transfer to oocytes, is enormously increased from a somatic level, up to 100 times for *Sox2*, the oocyte germinal vesicle does not cause a global transcriptional enhancement of all genes. RNA-Seq analysis shows that most genes in a mouse somatic cell are not changed in transcription, some remaining at a high level and others remaining at a repressed level. A minority of genes that were active in somatic cells become repressed after transfer to oocytes and a smaller fraction undergoes a great enhancement of transcription. Thus the reprogramming of somatic nuclei by the oocyte germinal vesicle is highly selective (Jullien *et al.* unpublished). Those genes that are transcriptionally activated include ones that are strongly

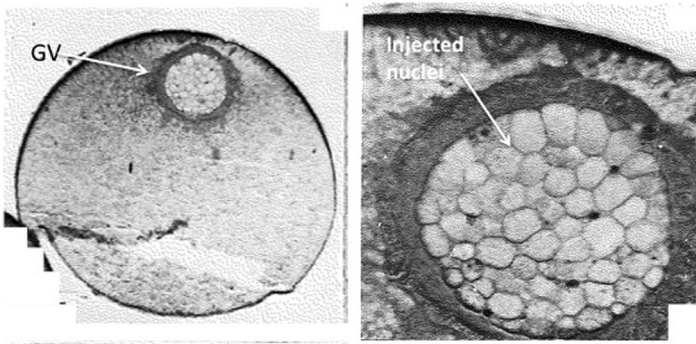


FIGURE 12. Multiple somatic nuclei can be injected into the germinal vesicle of an oocyte; whole oocyte (left) and germinal vesicle (right).

expressed and important in early mammalian development, including Sox2, Oct4, and Nanog. The germinal vesicle of an oocyte seems to be endowed with components that induce intense transcriptional activity, as seen in lampbrush chromosomes, for all genes that are accessible (Jullien *et al.* 2011). However some genes in somatic nuclei do not respond to the transcription-inducing conditions of the egg.

RESISTANCE TO REPROGRAMMING BY OOCYTES

To me, resistance to transcriptional activation is now the most interesting aspect of nuclear reprogramming. This increasing resistance associated with development seems to reflect the remarkable stability of cell differentiation (Fig 11). Hardly ever does a cell of one specialised type switch to another cell-type, or produce daughter cells that do so. Resistance to reprogramming is also evident in cell fusion experiments (Blau *et al.* 1983), and even more so in iPS work (Yamanaka 2012). The transplantation of mammalian somatic nuclei containing a repressed X chromosome has identified one kind of molecule responsible. Mouse embryo fibroblasts containing an inactive X chromosome are highly resistant to the transcription of these genes after nuclear transfer to oocytes. However, the nuclei of mouse embryo blastodisc cells that also contain an inactive X chromosome are strongly reactivated transcriptionally by oocytes. This difference between blastodisc and adult nuclei has turned out to be attributable to the chromosomal component macroH2A whose removal or inactivation in adult mouse embryonic fibroblast (MEF) nuclei results in transcription

of pluripotency genes (Pasque *et al.* 2011a). At present, we envisage the female mammalian X inactivation process as a set of steps that progressively stabilise the inactive state. Thus, as development and cell differentiation proceed, successive levels of inactivation, involving histone modifications such as H3K27Me2/3 and macroH2A absorption into chromatin, and finally methylation of DNA, cause a gene to become stably repressed and highly resistant to reprogramming (Pasque *et al.* 2011b).

To analyse other ways in which genes become resistant to reprogramming, two experimental routes are likely to prove useful. One is to progressively remove components of isolated nuclei, and then test their transcription in injected oocytes until resistance is lost (Halley-Stott, unpublished). This procedure is proving successful in depleting nuclei of all RNA including non-coding RNA. Increasing concentrations of NaCl with Triton can progressively deplete isolated nuclei of chromosomal proteins. If resistance can be restored by adding back defined fractions of released proteins, this could lead to the identification of those chromosomal proteins that confer resistance on individual genes. Another potentially valuable experimental approach is to overexpress, by mRNA injection to oocytes, those enzymes that add or remove modifications to histones. It should then be possible to relate a particular histone or other chromosomal modification to the resistance of a gene to reprogramming by oocytes. By these methods, there is a prospect of understanding, in reasonable detail, the mechanisms of nuclear reprogramming and resistance in nuclei transplanted to amphibian oocytes.

OVERVIEW AND PROSPECTS

The process of nuclear reprogramming by eggs and oocytes can be seen as a conflict between the cytoplasm of an egg whose components are designed to promote rapid DNA replication and then transcription, and the components of differentiated cell nuclei whose function is to maintain a stable state. The cytoplasm of an egg is specially designed to activate the highly condensed and specialised nucleus of sperm, with 100% efficiency. Not surprisingly, the same components are effective at activating the nucleus of a somatic cell. The difference is that a somatic cell nucleus has become, during the process of cell differentiation, highly resistant to activation by egg cytoplasm in a way that is different from sperm nuclei. These nuclei of differentiated cells are provided with molecules that stabilise their differentiated state and resist reversal or rejuvenation. If differentiated cell nuclei could be too easily switched to an embryonic

state, this could permit the reversal of differentiation and lead to cancer and other defects.

The experimental work described here has centred on the use of amphibian eggs and oocytes because of the abundance of material and ready availability offered by them, an advantage that was very clear to developmental biologists up to the 1950s. The general principles that have emerged from work on amphibia seem also to apply to mammals and other vertebrate species. A full understanding of nuclear reprogramming by amphibian eggs and oocytes may well facilitate nuclear reprogramming in mammals including humans, and hence contribute to the eventual therapeutic application of cell replacement.

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