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Nuclear transplantation and the conservation of the genome in development.

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When starting my graduate work in the 1950s, it was not known whether all different kinds of cells had the same set of genes in the same organism. It had indeed been suggested by Weismann (1892) that genes no longer required in development might be lost or permanently inactivated. For example, skin genes might no longer be needed for the lineage which gives rise to the brain, and the permanent inactivation or loss of genes no longer required in development could help direct cell differentiation in desired directions. Rauber (1886) published a paper describing the implantation of a frog nucleus into a toad egg and vice versa. He was curious to know what would happen in such an experiment, but he merely reported that development did not take place. It is unclear

whether he actually did such an experiment or not. It was clear that the ideal experiment would be one in which the nucleus of a specialized cell is injected into an unfertilized egg whose own chromosomes had been removed. The clear question was whether the nucleus of a cell which had embarked on one pathway of differentiation could nevertheless support development of other, unrelated cell-types. The first real success in transplanting living nuclei from one cell to another was achieved by Briggs and King in 1952. They showed that the blastula nucleus of a *Rana pipiens* egg could be transplanted to the enucleated egg of the same species and, in a significant number of cases, they were able to obtain swimming tadpoles. They also reported, in the same paper, that if they took the nucleus from a more advanced embryo, for example from a neurula embryo, the same experiment was not successful. They concluded, quite reasonably, that some change had occurred during early development such that the neurula nucleus was not longer able to substitute for the egg and sperm nuclei of a zygote.

When starting my graduate work, my supervisor, Dr M. Fischberg, advised me to repeat the experiments of Briggs and King but to use the frog *Xenopus* laevis instead of *Rana pipiens*. He took this view for two very good reasons. First, *Xenopus* embryos can be

grown to sexual maturity in less than a year, whereas the same process takes up to four years in *Rana pipiens*. Second, *Xenopus* frogs can be induced to lay eggs throughout the year, following hormone injection, whereas *Rana pipiens*, like most European frogs, lay eggs only in the spring of each year. Experiments with *Rana*, therefore could not be done for ten months out of each year. My view, at that time, was that if I could make nuclear transfer work in *Xenopus*, I might expect that either my results would reproduce those of Briggs and King, or that I might get more successful nuclear transfer embryo development. In either case there would be an opportunity to investigate the mechanisms that follow nuclear transfer and it was even possible that I might get more normal development than Briggs and King using nuclei of differentiated cells.

There were two major obstacles in achieving nuclear transfer in *Xenopus*. The first was that *Xenopus* eggs are surrounded by an extremely elastic jelly surrounding the membrane that encloses the egg, and this viscous jelly turned out to be impenetrable by even the sharpest of micro-needles (Fig. 1). The second problem was that the method of enucleation of the egg used by Briggs and King was not successful in *Xenopus* whose eggs suffered so much damage by cautery or physical attempts to remove the nucleus that they could

not be used for such experiments. The solution to these problems emerged, more by good luck thant judgement, within a year. First, an ultraviolet light used for microscopy turned out to be very effective at killing the egg mitotic chromosomes which, fortunately, were located on the surface of the egg. Ultraviolet light penetrated an egg only to a depth of about 30μ, and did not therefore significantly harm a *Xenopus* egg with a diameter of 1200μ. Had chromosomes not been in this position they could not have been killed by this means. The second piece of exceptionally good fortune was that the wavelength of the ultraviolet light used not only killed egg chromosomes, as might be expected, but also denatured the elastic properties of the jelly surrounding the egg so that, at the right dose, a needle could penetrate the egg without damaging the egg itself. Even then, I had to make a special micro-forge so as to put a sharp point on a micropipette that was also small enough to break a donor cell without damaging its nucleus (Fig. 2). In retrospect, this last piece of good fortune was not entirely surprising. After all, sperm enter the egg without causing damage.

Another piece of exceptional good fortune, or wisdom on the part of my supervisor, resulted in a novel genetic marker being discovered for *Xenopus*. It happened, at that time, that he had a

student, Sheila Smith, studying haploid development for which it was necessary to fertilize enucleated eggs and so to obtain haploid cells which were known to be characterized by a single nucleolus (which contains the ribosomal DNA encoding genes) in each cell, whereas normal diploid embryos usually have two nucleoli representing the two sets of ribosomal genes, in each nucleus (Fig. 3). It was known that haploid embryos do not survive long enough to commence feeding and die as crippled embryos. The student concerned found that, unexpectedly, she could obtain normal embryos which had only one nucleolus and which were diploid. Fortunately, my supervisor did not follow the normal route, when the results are unexpected, which would have been to ask the student to re-make all their solutions and start the experiment all over again with a new set of eggs. He asked the student to go through the whole *Xenopus* colony and see if she could find a female which had this ability to lay eggs whose derived embryos were diploid with only one nucleolus. Such an animal was, surprisingly, discovered and was the stock from which the well-known one-nucleolated diploid embryos (using the anucleolate mutant) were obtained (Elsdale *et al*., 1958). Thus it was possible to carry out a nuclear transfer experiment such that an unfertilized *Xenopus* egg could be harmlessly penetrated and, most

importantly, a genetic marker could be used to demonstrate unequivocally that the egg chromosomes had been killed and that any resulting embryos did indeed result from the genetic material of the transplanted nucleus.

It soon became apparent that, when I transplanted nuclei from the neurula stages of embryos as used by Briggs and King, I found no significant decrease in the normality of development (Gurdon 1960). This gave strong encouragement to continue these experiments using cells that were progressively more differentiated. I focussed on the endoderm lineage of embryos because the cells of this cell-type are very large and easy to handle because of their large content of yolk. This made it possible for me to eventually transplant nuclei from larval intestine cells, derived from the endoderm, and to achieve the transplantation of a nucleus from a cell which had become committed to a particular type of cell differentiation. I had been able to carry out a large number of such experiments and the resulting embryos, from the intestinal epithelium, had begun to metamorphose into young frogs. At that point I had committed myself to a post-doctoral position in California, where I was asked to concentrate on bacteriophage genetics. I therefore left my young froglets in the hands of my supervisor Michael Fischberg who had just moved to

Geneva as a Professor of Zoology. He asked his technician to look after the froglets and grow them up to adulthood. By the time I returned to Oxford from my postdoctoral period in California I went back immediately to the embryological work which had served me well. By this time the intestinal epithelium-derived frogs had reached sexual maturity and a considerable number of these frogs could be induced to lay eggs as females or fertilize eggs as males. In Oxford, I found that the intestine-derived frogs were indeed fertile and that their offspring showed no evidence of any defects beyond those which affect all laboratory-reared animals. This led to the end of that phase of my early career and I was able to publish a paper entitled, "Fertile-intestine nuclei" (Gurdon and Uehlinger, 1966). This gave the first decisive evidence that the nucleus of a specialized cell is totipotent.

I should add that, as cells become increasingly differentiated, their transplanted nuclei become progressively less able to support normal development of enucleated eggs, as clearly shown by the Briggs and King (1952) experiments. I found dramatic differences between nuclei from the same donor embryo in the serial nuclear transfer clones made from them (Fig. 3). This was probably due to

damage (possibly random) sustained in the first nuclear transfers as shown by Di Berardino and Hoffner (1970).

At that time I was subject to considerable criticism. It was reasonably thought that a young graduate student was most unlikely to be able to overturn the conclusions from very well-established and highly respected workers in the field, namely Briggs and King. In all, it took about ten years for my results to be generally accepted. Critical to the acceptance of these results was the benefit of using a nuclear marker, i.e. the single nucleolus mutant discovered by my supervisor. This made it almost impossible to reject the results of my experiments. Therefore the conclusion was eventually reached that as cells undergo progressive differentiation, their nuclei nevertheless retain the ability to bring about, in combination with unfertilized egg cytoplasm, almost all cell-types which characterize an individual. This conclusion is now generally accepted for all animals and plants. However, it took nearly 40 years for the same conclusions to be reached with mammalian nuclear transfer (Campbell *et al.* 1996; Wilmut *et al.* 1997). The reason it took so long for the *Xenopus* experiments to be reproduced in mammals was, at least in part, due to the decision by those attempting nuclear transfer in mammals to use fertilized recipient eggs that were subsequently enucleated

(McGrath and Solter, 1984). In *Xenopus,* and indeed in *Rana pipiens*, unfertilized eggs had been used to receive transplanted nuclei. Once fertilization has taken place it is much harder to replace the zygote nucleus with a somatic cell nucleus. In the end, and as it now is, the original design of Amphibian experiments needed to be repeated exactly in mammals and, under these conditions, and as done in the sheep work referred to above, nearly all mammals can now be used for successful somatic cell nuclear transfer.

It is conceivable that the enucleation of *Xenopus* eggs by ultraviolet light gave them some advantage compared to enucleation of *Rana* eggs by microsurgery. Ultraviolet light destroys DNA but will leave most of the proteins which are associated with chromosomes, or present but unassociated with chromosomes in the nucleus, still intact. It has now been shown that there is a dispersal of some nuclear proteins into the cytoplasm at mitosis (Martínez-Balbás *et al.* (1995). If such components are important in permitting successful somatic cell nuclear transfer, they might well have been removed or destroyed by the physical removal of an egg nucleus or its chromosomes, whereas they might remain intact and available for reprogramming the nucleus after ultraviolet destruction of DNA.

Looking back, I was extremely fortunate to be supervised by Michael Fischberg and directed onto a really important problem very early in my career. He was extremely generous in giving me every facility to use in his laboratory. He was also generous enough to let me publish most of my work as a single author without attaching his name to the work that I did in his laboratory. Nowadays, he, in his capacity as Head of the research group, would certainly have expected to be a subsidiary author on all of my early *Xenopus* nuclear transfer work. I am however glad that he was first author on the initial paper describing success with nuclear transfer in *Xenopus* (Fischberg et al. 1958).

One might ask, "What next?" I have devoted the rest of my scientific career in attempts to understand the mechanism of nuclear reprogramming which takes place after nuclear transfer in *Xenopus* (e.g. Jullien *et al*. (2011). We would very much like to know what are the molecular events which permit this remarkable reprogramming of the somatic nucleus to take place efficiently and quickly. It was some half-century after these early *Xenopus* nuclear transfer experiments that Takahashi and Yamanaka (2006) announced the amazing result that they could reprogram a small percentage of somatic cells back to an embryonic state by overexpression of

selected transcription factors. This celebrated work has rightly captured the interest and enthusiasm of almost all laboratories and has led to the founding of numerous "regenerative medicine" laboratories in many if not most countries. It would, however, be unrealistic to use nuclear transfer to eggs as a route towards regeneration of replacement cells in humans, because human eggs are hard to obtain in any number.

The success of the Takahashi/Yamanaka procedure is already beginning to reach the point where clinical benefit can be made available to humans, in particular with respect to the replacement of the pigmented epithelium of the eye for patients with age-related macular degeneration [Ramsden *et al.* 2013].

If we could understand the details of how the egg cytoplasm can so efficiently reprogram a somatic cell nucleus, this could greatly improve the success of reprogramming accessible somatic cells such as those of skin or blood to give large numbers of new cells of many different kinds. In this way, the reprogramming of somatic cells is likely to be of enormous benefit to humans for the alleviation of disease or ageing.

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Figure legends

Fig. 1 ~~Even/not~~Not even a sharpened micropipette can puncture the highly elastic jelly that surrounds *Xenopus* eggs.

Fig. 2 A microforged injection pipette had to be sharp enough not to damage a recipient egg, but with a sufficiently smooth opening not to break a nucleus. It had also to be small enough to break the donor cell plasma membrane.

Fig. 3 Development of the anucleolate mutant in Xenopus (from Elsdale et al. (1958). Heterozygotes develop entirely normally into fertile adult frogs. Homozygote mutants with ribosomal RNA genes die as swimming tadpoles before feeding; they develop this far by use of maternal ribosomes in the egg.

Fig. 4 Serial cloning of a transplanted endoderm nucleus yields dramatically different survival of embryos (Gurdon, 1962). Twenty transplant-embryos were obtained from endoderm nuclei of an original macular-response stage tadpole. When these embryos had become late blastulae, three were used to provide nuclei for serial transplantation. The three resulting clones differ strongly from each

other. About 50 transplantations were made for each of the four series. [note remove existing text from this figure]

Fig. 5 Michael Fischberg (19 -) The first nuclear transplantation experiments were done when he was a lecturer in the Zoology Department at Oxford in 1956