Nuclear reprogramming in eggs

John Gurdon

Early nuclear transfer experiments

I must have been born with a strong attraction toward, and possibly even an aptitude for, doing things on a small scale. I remember that, at an early age, I spent many months making a three-masted sailing boat with rigging in a half-walnut shell. As a student, I took a job dissecting the genitalia of tiny moths (*Lithocolletis*), the larvae of which live in between the two layers of a leaf. When I started my PhD work at Oxford, England, my supervisor Michael Fischberg had the wisdom to allocate to me the small-scale problem of making nuclear transplantation work in *Xenopus laevis*. But my contemporaries asked me why I wanted to start my scientific career doing an experiment that had already been done and published. Briggs and King[1] had shown in 1952 that they could obtain normal tadpoles by transplanting nuclei from blastula cells to enucleated eggs in the frog *Rana pipiens*. But it seemed clear to me that the most important experiment, transplanting nuclei from differentiated cells, had yet to be done. Whatever way that experiment turned out, it was sure to be interesting and worth further analysis. Furthermore, unlike other amphibian species traditionally used for embryology that produce eggs during only two months of the year, *Xenopus* could be stimulated to produce fertile eggs at any time throughout the year[2]. Fischberg was also aware that the *Xenopus* life cycle of less than one year offered the possibility of using mutants—unrealistic in the much longer life cycle of most other amphibians—even though no *Xenopus* mutants existed at that time.

My first attempts to transplant nuclei in *Xenopus* were completely unsuccessful, because the *Xenopus* egg, unlike those of other amphibians, is surrounded by an extremely elastic membrane and jelly layer that make penetration by a micropipette impossible. By good fortune, Fischberg had just acquired a new ultraviolet illumination source for his microscope. We found that the wavelength of ultraviolet light from this source damaged the chromosomes, which are located on the surface of the *Xenopus* egg, thereby selectively enucleating it; ultraviolet light does not penetrate or damage the egg to a depth of more than 50 µm in an egg of 1,200 µm in diameter. Even more fortunate was our finding that this ultraviolet source denatured the egg's outer coverings enough to make it harmlessly penetrable by a suitable micropipette, which I obtained by designing and making a new microforge able to put hypodermic tips on the ends of glass microneedles.

Within six months of starting my PhD work in 1956, I had already obtained feeding tadpoles derived from transplanted nuclei of embryonic cells. Fortuitously, a student of Fischberg had just discovered a female frog in his laboratory stock that gave an unusual distribution of nuclei in embryos derived from its eggs[3]. This was later shown to be a deletion of the *18s* and *28s* ribosomal RNA genes—the so-called 1-*nu* mutation[4]. Fischberg realized that this could be used as a genetic marker for nuclear transfer experiments. The viable heterozygous embryos or tadpoles carried the 1-*nu* mutation, which gave crucial proof that 1-*nu* nuclear transplant embryos were derived from the transplanted nucleus and not from a failed enucleation of the recipient egg (**Fig. 1**).

Soon after we started our *Xenopus* experiments, Briggs and King[5] published another paper showing that the nuclei of embryos about one day older than the blastulae they had used in their previous work were unable to elicit normal development of nuclear transplant embryos. They concluded that, as cells differentiate, their nuclei undergo stable changes such that the nuclei are no longer totipotent by the criterion of nuclear transplantation. This conclusion was later considered incorrect in the light of the *Xenopus* experiments. While I was working as a postdoctoral fellow in a different field, Fischberg and his assistant Vreni Uehlinger had maintained and tested the fertility of the nuclear transplant frogs that I had made from the nuclei of functional intestinal epithelium cells. Most of these frogs were normally fertile and generated entirely normal offspring[6]. Some years later, my lifelong colleague Ronald Laskey and I were able to create normal swimming tadpoles, but not adults, by transplanting nuclei from a range of adult frog tissues, thereby complementing the derivation of fertile adult frogs from feeding tadpoles[7]. The normality and fertility of the frogs that carried the 1-*nu* nuclear marker established the general principle that the process of cell differentiation is not accompanied by any loss or stable inactivation of genes. The rearrangement of the genome in antibody-forming B cells of the immune system is a special exception. For reasons not yet understood, the *Rana pipiens* experiments of Briggs and King did not yield the same results, but the principle of genome conservation during cell differentiation has been subsequently reinforced by experiments with adult frog skin, using antibodies to keratin to prove the specialized state of donor cells[8].

As a result of my nuclear transfer experiments, speculation arose about the possibility of making human clones like the ones I had described for *Xenopus*[9]. The famous American news writer Walter Cronkite (who died this year) came to interview me in Oxford and asked how long it would take for cloning to work in mammals and hence in humans. I guessed anywhere from 10 to 100 years.

Nuclear reprogramming

The differentiated state of cells is remarkably stable; hardly ever, and maybe never, does a differentiated cell or its daughters switch from one differentiated type, such as intestine, to an unrelated one such as brain or muscle. It was already evident from the

John Gurdon is at the Wellcome Trust/Cancer Research UK Gurdon Institute and Department of Zoology, University of Cambridge, UK.
e-mail: j.gurdon@gurdon.cam.ac.uk

Figure 1 The early days of nuclear reprogramming. (**a**) Clones of albino *Xenopus* frogs (all male) obtained by transplanting nuclei from an albino embryo into eggs of the wild-type dark green female shown at the top of the image. All of the clones are genetically identical, like identical twins. (**b**) The author in 1960 in the room in which the *Xenopus* frogs and clones were maintained.

early *Xenopus* nuclear transfer experiments that the egg cytoplasm induces a substantial reversal of differentiation. Indeed, the conversion efficiency of intestine to functional muscle and nerve is about 30%; nearly one-third of transplanted intestine nuclei can generate tadpoles able to respond to an external stimulus by swimming (**Fig. 2**)[10]. The egg uses its natural properties to cause a reversal of differentiation as it does after fertilization by sperm. Like the nuclei introduced by the nuclear transfer experiments, a mature sperm nucleus enters an egg and is reprogrammed to produce all the cells of an animal's body.

An early aim of the *Xenopus* nuclear transfer experiments was to identify these reprogramming molecules and mechanisms. A good start was DNA replication. In *Xenopus*, the egg and sperm pronuclei start very intense DNA replication within 20 minutes of fertilization, and the whole chromosome set is completely replicated within the next 20 minutes. My first graduate student, Christopher Graham, showed that nondividing adult frog brain nuclei initiate DNA synthesis within 20 minutes after their transfer to eggs[11]. Subsequently, Laskey developed a cell-free system derived from *Xenopus* eggs to identify molecules that initiate DNA synthesis and to work out a mechanism that ensures only one round of chromosome replication during each cell division[12]. He later developed from this work a cancer diagnostic test based on the DNA replication control molecule MCM; this test is now being validated for clinical screening for breast cancer, colorectal carcinoma and others[13,14]. This was an unexpectedly useful result of his earlier nuclear transfer work.

To analyze nuclear reprogramming at the transcriptional level is not easy. This is, in part, because *Xenopus* embryos are transcriptionally quiescent until they reach the 4,000-cell stage. It is therefore not clear when somatic nuclei are transcriptionally reprogrammed: immediately after nuclear transfer, or later in development? My colleague Hugh Woodland and I established, by the crude methods then available, that at least those nuclear transplant embryos that reach the blastula stage of development have had their pattern of transcription reversed by the egg cytoplasm from that of an adult cell to that of an embryo[15]. A current direction of egg reprogramming work is to inject somatic nuclei or DNA[16,17] into the so-called germinal vesicle of growing eggs (described as oocytes) during the first meiotic prophase. This procedure causes the pluripotency genes of mammals to be efficiently activated by *Xenopus* oocytes[18,19]. As oocytes are completely inactive in DNA replication, in contrast to eggs, it has been possible to identify some of the reprogramming molecules required for transcriptional activation. These include Tpt-1 (ref. 20) and an embryo-specific linker histone (J. Jullien, L. Astrand and J.G., unpublished data).

Cloning in mammals

Why did it take nearly 40 years for nuclear transfer to be achieved in mammals, when the technique used is essentially the same as that originally developed for frogs? An early report by my student Derek Bromhall showed that nuclei of embryonic cells transplanted to rabbit eggs do synthesize DNA[21]. For reasons I have never fully understood, the next major attempts to transplant nuclei in mammals used fertilized eggs as recipients, rather than unfertilized eggs as had proved successful in frogs. For example, McGrath and Solter[22] found that even the nucleus of a four-cell embryo does not support substantial development when transplanted to a fertilized, but subsequently enucleated, egg. Wilmut and Campbell[23,24] were the first to obtain a normal, fertile adult mammal—a sheep—by transplanting nuclei from the cells of an adult. This breakthrough extended the earlier results with amphibians by showing that a normal adult mammal can be obtained by transplanting the nucleus of an adult cell. Soon, somatic cell nuclear transfer in mice was achieved[25], and, through careful technical refinements, it has now been achieved in many mammalian species[26]. An elegant reinforcement of these conclusions came from the successful transplantation of nuclei from antibody-forming B cells (which have a rearranged genome)[27] and from differentiated neural cells[28,29].

Compared to the great stability of differentiation in normal cells, it is remarkable how effectively this state can be reversed by nuclear transfer to eggs. The potential usefulness of this became especially clear with the amazing discovery of Evans and Kaufman[30] that lines of indefinitely proliferating embryonic stem cells can be derived from mouse embryos, and that these cells can be made to differentiate into all cell types found in the adult. The combination of nuclear transfer and embryonic stem cell technology offers a clear possibility of cell replacement. In principle, easily accessible cells such as skin cells could be used to derive new (rejuvenated)
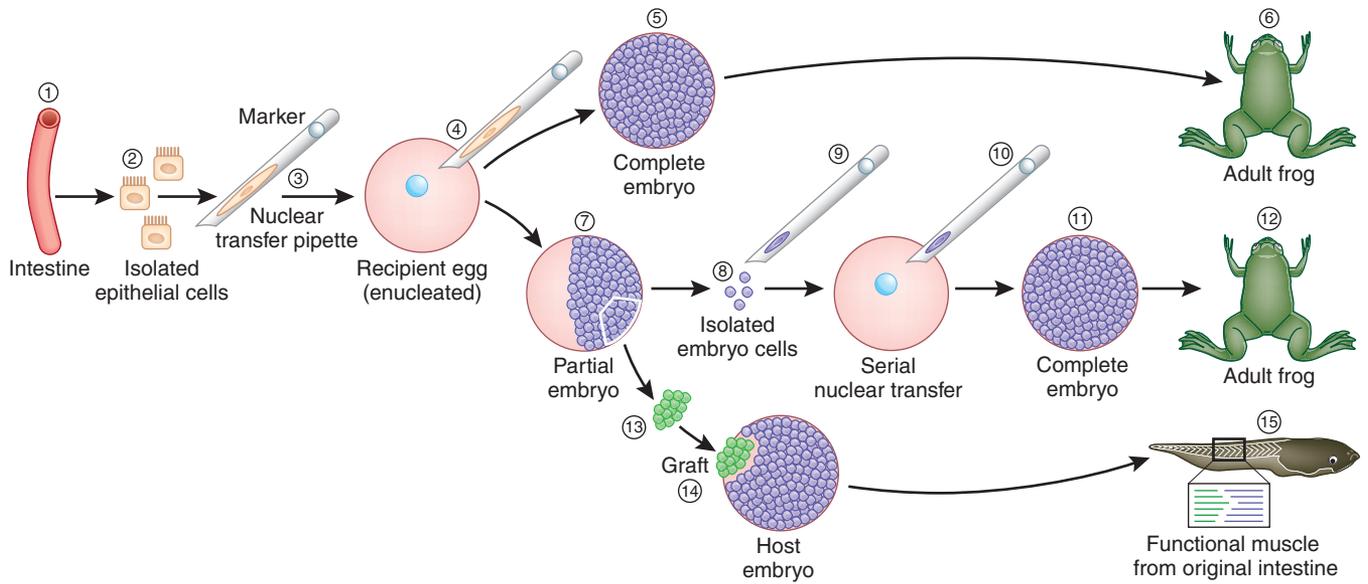
Figure 2 In this example, a piece of intestine is cut out (1), and brush-border epithelial cells are dissociated to use them for nuclear transfer (2). A single donor cell is sucked into a narrow micropipette (3) to break the plasma membrane, but not the nucleus. The recipient egg is enucleated by ultraviolet irradiation, the micropipette is injected into the egg and the donor nucleus is ejected from the micropipette (4). Some injected eggs form complete embryos (5), some of which develop into adult frogs (6). Some injected eggs form partial embryos (7), cells from which can also be isolated, collected and have their nuclei transferred into enucleated eggs (8–10). This process is termed serial nuclear transfer and can also result in the formation of full embryos and the birth of live frogs (11,12). If a small piece of a partial embryo (13) is labeled and transplanted into a host embryo (14), the resulting tadpole (15) has parts derived from the graft. Here, a tail muscle has cells with a nucleus that originated from the intestine of the original donor and cells from the host embryo. Experiments like this have shown that up to 30% of the original intestinal epithelial cells have nuclei that can generate functional muscle and nerve cells, highlighting the high efficiency of nuclear reprogramming by eggs.

cells of any desired kind and thus provide an individual with cells of his or her own genetic constitution without the need for immunosuppression.

Mechanisms

The simplest process by which an egg or oocyte could reprogram a somatic nucleus seems to be as follows. During its formation, the egg builds up a high concentration of reprogramming molecules, such as the linker histones B4 and H1foo. By the combination of mass action and replacement of chromosomal proteins, egg molecules displace or replace repressor molecules associated with nonexpressed genes in somatic cells. In frogs and mammals, about 5 and 20 hours pass, respectively, between the time of nuclear transfer and new gene transcription. So there is ample time for this exchange to take place, and almost all transplanted nuclei should elicit normal development if this were the only mechanism involved. However, normal development to the adult stage occurs only rarely with transplanted nuclei from specialized cells.

A similar conclusion can be drawn from two other experimental procedures (apart from nuclear transfer to eggs). One is induced pluripotency, in which transcription factor overexpression can cause specialized cells to become embryonic[31] or to switch from exocrine to endocrine cell types in the pancreas[32]. In the earliest transcription factor overexpression experiments of Weintraub[33], many, but not all, kinds of cells could be induced to become muscle by overexpression of the muscle-specific transcription factor MyoD. The second procedure is cell fusion, a methodology that opened the way to the discovery of monoclonal antibodies. For example, a differentiated chick erythrocyte[34] or a human amnion cell[35] can be fused to a fibroblast or a multinucleate muscle fiber, respectively, and be reprogrammed to express genes characteristic of the host cell. But in both of these cases, only a minority of the fused or factor-treated cells undergo the change, and a high degree of selection is required to isolate the reprogrammed cells. So, as with nuclear transfer to eggs, there is considerable resistance to the reversal of the differentiated state.

Mechanisms of reprogramming are of great current interest. Induced pluripotency is thought to be a stochastic process in which only a small minority of the treated cell population receive the optimal amount of transcription factors or are in a particular state to respond to them[36]. In cell-fusion experiments[37], mutant cell lines, such as *Eed*^{-/-} cells (embryonic ectoderm development protein–deficient cells), are being used to test the involvement of known gene repressor molecules[37]. In the case of nuclear transfer to eggs, we recently discovered a phenomenon of epigenetic memory in which some nuclei continue to express their specialized-cell genes long after nuclear transfer, apparently ignoring the effect of egg-reprogramming molecules[38]. A variant histone, H3.3, seems to have a role in the conservation of a differentiation pathway after nuclear transfer[38] and, therefore, in providing resistance to the reprogramming effect of egg cytoplasm. It has been suggested that, as genes such as those encoding Oct4 become repressed during cell differentiation, they become accessible to reprogramming factors for progressively shorter amounts of time[39], accounting for the decreasing success of nuclear transfer as cells become more differentiated and for the low success rate of induced pluripotent stem cells.

A major advance in the future will be to identify the basis of the stability of the differentiated state of cells. We could then understand how egg-reprogramming factors work. We might be able to reprogram somatic cell nuclei much more efficiently, using the endogenous molecules and mechanisms employed by eggs after fertilization, and hence eventually achieve cell replacement in humans without immunosuppression.

COMMENTARY

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