Somatic cell nuclear transfer: origins, the present position and future opportunities

Ian Wilmut, Yu Bai and Jane Taylor

MRC Centre for Regenerative Medicine, University of Edinburgh, BioQuarter, 5, Little France Crescent, Edinburgh EH16 4UJ, UK

Nuclear transfer that involves the transfer of the nucleus from a donor cell into an oocyte or early embryo from which the chromosomes have been removed was considered first as a means of assessing changes during development in the ability of the nucleus to control development. In mammals, development of embryos produced by nuclear transfer depends upon coordination of the cell cycles of donor and recipient cells. Our analysis of nuclear potential was completed in 1996 when a nucleus from an adult ewe mammary gland cell controlled development to term of Dolly the sheep. The new procedure has been used to target the first precise genetic modification into livestock; however, the greatest inheritance of the Dolly experiment was to make biologists think differently. If unknown factors in the recipient oocyte could reprogramme the nucleus to a stage very early in development then there must be other ways of making that change. Within 10 years, two laboratories working independently established protocols by which the introduction of selected transcription factors changes a small proportion of the treated cells to pluripotent stem cells. This ability to produce ‘induced pluripotent stem cells’ is providing revolutionary new opportunities in research and cell therapy.

1. Introduction
As we know it today, ‘nuclear transfer’ involves the transfer of the nucleus of a donor cell into an oocyte or early embryo from which the chromosomes have been removed. In some cases, the transferred nucleus successfully controls development to term of the reconstructed embryo. Nuclear transfer of this type only became technically feasible in amphibians around 1950 and in mammals some 30 years later. There are many variations between species in the details of the techniques used to make these changes, to account for biological differences between species and because of the experience and habits of the laboratory carrying out the experiment. Since the quality of optical systems and micromanipulators made precise, reproducible micromanipulation possible the challenge has been to learn how to carry out nuclear transfer in such a way that some of the reconstructed embryos are able to complete development. In turn, this has revolutionized our understanding of developmental biology and created new opportunities in research and medicine.

The suggestion of transferring a nucleus from a somatic cell into an enucleated oocyte arose first during a search for the mechanisms that regulate embryo development. In the late nineteenth century, it was generally understood that successive generations of a species are extremely similar under the influence of regulatory mechanisms contained within the germplasm. Today, we would say that these regulatory mechanisms reside within the chromosomal DNA. Although these factors were not then understood, this was an aspect of biology that was under active consideration and experimentation. One hypothesis, discussed by August Weissmann in 1885 [1], was that during development, sections of the germplasm are lost so that the fate of a cell is determined by the fraction of the germplasm that remains. This led to the suggestion that a nucleus from a differentiated cell would not be able to control development of a complete offspring, because it would not have all of the necessary instructions. Accurate tests of this hypothesis were not possible for almost a
century, but in the meantime a series of experiments were carried out that provided important background information and led to the development of the techniques for micromanipulation of mammalian embryos that were required for nuclear transfer.

2. Early experiments

When the cells of early cleavage stage embryos were separated, it was found that each of the cells was able to support development to term so demonstrating that each cell has all of the factors that regulate development. This ability was shown first in sea urchin by Dreisch (1891 described in [2]). In subsequent experiments, similar observations were made in salamander by Spemann [3], ungulates by Willadsen [4] and mice by Rossant [5]. This ability was found to be limited to very early cleavage stages, but the factors that imposed this limitation were believed to vary between species. In sea urchin and salamander, it was thought that separation of the cells was too damaging for them to survive. By contrast, in the mouse the ultimate limitation was apparently the inability of embryos derived from one blastomere from 8-cell embryos to form a normal inner cell mass. As a result, they were rarely able to develop the full range of tissues and organs [6]. A different estimate of developmental potential was obtained when cells from early embryos were aggregated with tetraploid embryos. Tetraploid embryos do not have the potential to develop to term, but complete early development and early post implantation development. Live offspring were born following aggregation of single cells in tetraploid embryos. The resulting offspring were made up exclusively of diploid cells showing that these cells had the ability to form all tissues and that tetraploid cells did not contribute to the offspring [7].

Hans Spemann is often considered the father of cloning because of an experiment in which he used a hair from his baby daughter to control the location of a nucleus within a developing salamander embryo. He tied a ligature in such a way that the nuclei were restricted to a portion of the cytoplasm, creating an anucleate fragment. The nucleated segment of the embryo was allowed to divide several times before the ligature was loosened in order to allow one nucleus to move into the anucleated fragment of cytoplasm which had not divided. Ultimately, the two sections of the embryo each gave rise to a normal salamander showing that the nucleus that had divided several times before release into the anucleate fragment had not lost key regulatory factors, at least during that early stage of development (described in [2]). A few years later, he was the first person to describe what we would now recognize as nuclear transfer, describing it as a ‘fantastical experiment’ [3].

3. Nuclear transfer in amphibians

The fantastical experiment was first attempted in amphibians because the comparatively large size of the eggs makes micromanipulation easier and large numbers of eggs and embryos may be obtained relatively easily. In the first studies, which were in the North American leopard frog Rana pipiens, Briggs & King [8] described a method for transfer of amphibian nuclei and reported that tadpoles developed following transfer of nuclei from early cleavage stage embryos to enucleated eggs. Later they also noted that nuclei taken from the presumptive medullary plate of late gastrulae were not limited to participation in neural differentiation, but also participated in mesodermal and endodermal differentiation [9]. Commenting upon these results the authors wrote ‘this suggests a progressive specialisation of nuclear function during cell differentiation. Concerning its nature there is as yet very little known. How it may differ in different tissues, whether it depends upon alterations in chromosomal or other aspects of nuclear function, and the question of correlated changes in cytoplasmic elements are among the problems remaining to be worked out’ [9, p. 324].

In all experiments in which the aim is to produce cloned offspring, it is essential to have good markers to confirm the genotype of the offspring. Differences between the donor and recipient animals in skin pigmentation were accepted as sufficient evidence in early studies, but in his experiments Gurdon took advantage of a mutation which reduced the number of nucleioli in the donor cells. This allowed him to identify the source of the nucleus which had supported development [10]. He noted that the pronucleus derived from the oocyte very rarely contributed to the developing embryo and the rare cases in which this happened could easily be identified and eliminated from the dataset. His experiments were carried out in Xenopus laevis the South African clawed frog. This eliminated the need for enucleation, saving time and reducing the stress imposed upon the oocyte during nuclear transfer in this species.

Once repeatable protocols were established, developmental potential of the transferred nucleus and factors that influenced development of the reconstructed embryo were analysed. It was established that efficiency decreased as the transferred nuclei were obtained from later stages of development [11], although there were differences between the two species in the pattern of the change.

Initially, development was only obtained with nuclei taken from early cleavage stages, but refinements to the protocols, including the use of different medium to handle the nuclei and of serial nuclear transplantation, made it possible to obtain swimming tadpoles and mature adults following transfer of nuclei from fully differentiated cells [11]. Considering these and many other studies in amphibians, it was clear that the reversible nature of differentiation was incompatible with the suggestion that differentiation is brought about by loss of germplasm (chromosomal DNA) [1].

Despite years of research, it is still the case that nuclei from fully differentiated cells from tadpoles have supported development to sexually mature frogs and nuclei from adults support development of tadpoles, but no adult frog has formed following a single transfer of a nucleus from an adult frog. Serial transfer did yield adult frogs following transfer of nuclei from adults or from cultured cells. It is perhaps partly because of this limitation that the research in amphibians did not prompt research to assess the possibility of dedifferentiating cells in the laboratory. It should also be noted that at that time a second limitation was imposed by the inadequate understanding of the role of transcription factors in controlling cell fate. Research to change the phenotype of cells by introduction of specific proteins was initiated followed the birth of Dolly the first adult clone of an adult animal (see below), by which time several transcription factors were known to be essential for the normal function of mouse embryonic stem (ES) cells [12].
4. Nuclear transfer in mammals

(a) Technical aspects

The opportunity to attempt nuclear transplantation in mammals depends upon enabling techniques to obtain large numbers of oocytes or zygotes, to culture them in vitro during and after micromanipulation, and then to give them the opportunity to develop to term in a surrogate mother. This in turn depends upon an ability to control the oestrous cycle and induce animals to superovulate. By ca 1960, embryo recovery and transfer was routine in mice, rabbits, sheep, cattle and pigs. Given this expertise, it was then possible to contemplate experiments in nuclear transfer.

The first nuclear transfer protocol to yield reproducible results was in the mouse by McGrath & Solter [13,14]. Nuclei from zygotes and early cleavage stage embryos were transferred into enucleated zygotes. When nuclei were transferred into enucleated zygotes at the same stage of development as the donor the proportion of reconstructed embryos that developed to term was not significantly different from that of non-manipulated controls [13]. By contrast, when donor nuclei were transferred from 2-, 4-, 8-cell stages or inner cell mass embryos, there was a dramatic decrease in the development to blastocyst stage, such that no blastocysts formed following transfer of nuclei from 8-cell or inner cell mass cells [14]. The authors attributed this outcome to an inability of the recipient zygotes to ‘reprogramme’ the different pattern of expression of the recently observed imprinted genes and commented that these results suggest that ‘the cloning of mammals by simple nuclear transfer is biologically impossible’ [14]. By contrast, when nuclei from 4- and 8-cell mouse embryos were transplanted into enucleated 2-cell embryos, a proportion developed not only to blastocyst, but to term. The difference between these results emphasizes that recipient cytoplasm influences the development of cloned embryos. Nuclear transfer in those first experiments in the mouse involved removal of nuclei from the recipient zygotes or early cleavage embryos by micromanipulation and introduction of the donor nuclei by fusion of a karyoplast to the recipient embryos by inactivated Sendai virus. Since then a variety of different protocols have been established [15].

One major change in the protocol was to use oocytes enucleated at metaphase II of meiosis as the recipient cell [16]. This in itself imposed additional technical challenges in order to be able to remove the metaphase spindle without causing harm to the remaining cytoplasm, and following transplantation of the nucleus, to induce parthenogenetic activation. There is a great difference between species in the transparency of oocytes, from the relatively clear mouse embryo, reviewed by Mann & Bartolome [23]. To a greater extent, progress in defining these points was based on empirical analyses of factors that influence the development of cloned embryos and our understanding of the underpinning biology of reprogramming is still rather limited.

As the research was progressing, it became apparent that normal development was dependent on creating an embryo in which DNA replication and the regulation of gene expression are appropriate for normal development. One very significant advance was the recognition that, in turn, these are controlled by the coordination of cell cycle stage of donor cell and recipient oocyte [20–22]. More recent research has emphasized that appropriate epigenetic regulation of gene expression is critical for the subsequent development of the embryo, reviewed by Mann & Bartolome [23]. To a greater extent, progress in defining these points was based on empirical analyses of factors that influence the development of cloned embryos and our understanding of the underpinning biology of reprogramming is still rather limited.

In a number of earlier studies, it was noted that when recipient cells were oocytes at metaphase II, subsequent development was greater if the donor cells were early in the cell cycle rather than later [24]. With this combination, live mice were obtained following nuclear transfer from 8-cell stage embryos. The first clear analysis of the effect of varying the cell cycle phase of donor cell and the oocyte during nuclear transfer in mammals was carried out by Keith Campbell at the Roslin Institute [20]. His study also provided a molecular explanation for the effects. Blastomeres from 8-cell stage cattle embryos were used as nuclear donors after synchronization optimized conditions, it did not reduce subsequent development provided that exposure was for not more than 15 s [16]. Others have used staining with Hoechst to confirm successful enucleation [18]. The oocyte was stained with Hoechst, but examined first under differential interference contrast optics. An indication of the location of the spindle was provided by localized differences in the appearance of the cytoplasm and the location of the first polar body. A section of the cytoplasm believed to contain the spindle was aspirated before the oocyte was removed from the field of illumination. The aspirate was then examined under UV illumination to confirm the presence of stained DNA from both the first polar body and the spindle. If enucleation had not been successful, then either the process was repeated or the oocyte was discarded. More recently, different optical systems able to reveal the spindle have been introduced and used effectively, e.g. Oosight™ (Hamilton Thorne, Ltd.). This system enables high-contrast quantitative imaging of the spindle without the use of exogenous dyes or labels. These dyes can have a detrimental effect on egg quality, especially from primates. This new technology uses birefringence to visualize the chromosomes, and the successful generation of primate and human somatic cell nuclear transfer (SCNT) embryos has been attributed, at least in part, to this technological breakthrough (see below).

A variety of treatments have been used to activate oocytes, indeed there is a monograph [19] on parthenogenesis. There are differences between species in the most effective treatment. During fertilization, entry of the sperm induces the release of intracellular calcium in a series of pulses. None of the current methods of activation in routine use precisely mimic this effect, but rather they tend to induce one prolonged release of calcium. Nevertheless, exposure to ethanol, strontium or pulses of alternating current are all in routine use to induce parthenogenic activation in one or more species.

(b) Underpinning biology

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such that they could be used specifically in either G1/S phase or G2 phase [20]. In vitro matured bovine oocytes were enucleated 30 h after onset of culture approximately 6 h after the oocytes reached metaphase II. The reconstructed embryos were assessed for changes in transferred nuclei: including nuclear membrane breakdown, premature chromosome condensation and DNA replication. The level of maturation promoting factor (MPF) activity was assessed by measurement of histone H1 kinase activity [20].

A number of conclusions emerged from this study. When nuclei were transferred into oocytes at metaphase II, this was followed by nuclear membrane breakdown and chromosome condensation regardless of their stage of the cell cycle. During subsequent culture, the nuclear membrane reformed and chromosomal DNA was replicated, regardless of cell cycle stage of the donor. By contrast, when the nucleus was transferred into oocytes that had been activated, then the nuclear membrane remained intact and whether or not DNA replication occurred was determined by the stage of the cell cycle of the nucleus. That is, it occurred in nuclei at G1/S, but not G2 [20]. These observations provided an explanation for the low frequency of development of cloned embryos, but also suggested two different approaches to coordination of cell cycle stages in such a way as to lead to normal ploidy in the reconstructed embryo.

Pre-activated oocytes would provide an appropriate environment for nuclei at any stage of the cycle, because the nucleus initiates DNA replication as required. We termed the activated oocytes ‘universal recipients’ as they are believed to provide an appropriate environment for nuclei at any stage of the cell cycle. We went on to demonstrate enhanced development to blastocyst stage when nuclei from 16-cell stage embryo blastomeres taken at unknown stages of the cell cycle were transferred either into oocytes at metaphase II (21.3%) or pre-activated oocytes (55.4%). In retrospect, it seems likely that Willadsen used ‘universal recipients’, unknowingly, in his pioneering nuclear transfer experiments [17]. The sheep oocytes were used 40 h after induction of ovulation by administration of gonadotrophin to the donor ewes. As the ewes are expected to ovulate approximately 24 h later, the oocytes were 16 h old when manipulated and might well be judged to be aged and thus vulnerable to spontaneous parthenogenetic activation by changes in temperature or culture medium [25]. If the transferred nucleus was in S or G2 phase, then normal ploidy was only to be expected if the recipient oocyte had entered S phase such that MPF activity had declined and did not cause nuclear membrane breakdown leading to further DNA replication. In this situation, the nucleus determines whether or not the DNA is replicated. This response would be expected to lead to normal ploidy.

By contrast, oocytes at metaphase II would be appropriate recipients for donor nuclei awaiting DNA replication (G1). Unfortunately, cells in early mammalian embryos do not respond to the usual check points and this makes it difficult to obtain groups of cells in G1. The only opportunities are either to inspect the embryo very frequently in order to observe the moment of cell division or to arrest the embryos in mitosis for several hours before releasing them. In either case, the cells can then be used as nuclear donors during the time period when they are expected to be passing through G1 phase, without further assessment. These procedures are effective, but time consuming and tedious.

Emboldened by this new understanding, the group then went on to transfer nuclei from cultured cells derived from embryos. ES cells have not been derived from ungulate embryos despite the fact that a considerable effort has been made. During attempts to derive such lines, it was common experience that after a few passages there were no cells which resembled ES cells. The initial objective of the experiment was to transfer nuclei from the culture as this happened. The expectation was that as cells differentiated it would be more difficult to obtain offspring after nuclear transfer. ES cells are derived from the inner cell mass or epiblast, and the expectation was that the proportion of cloned embryos that developed would decrease as differentiation occurred. In this way, we would learn more about the developmental potential of cells in the culture.

Cells were derived from the embryonic disc of day 9 sheep embryos by selection of cells that resembled mouse ES cells. However, by passage 3, the cells were flattened and epithelial in appearance. By passage 6, they expressed cytokeratin and lamin A/C, markers of differentiation. Using the Universal recipient protocol, donor nuclei were transferred into enucleated oocytes between 4 and 6 h after their activation [26]. During the first season of the study, live offspring were obtained following transfer of nuclei from cells taken at passage 1, 2 and 3. During the following season, nuclei were transferred from cells taken at passage 6 and 11, but no offspring developed. However, nor were there any lambs following control transfer. As a result, the study was inconclusive.

However, during this second season, a different opportunity presented itself in the form of an epithelial line established from these cultures by our colleague Jim McWhir. Keith Campbell found that in response to serum starvation these cells exited the cell cycle and became quiescent (G0 phase). At this stage, they were candidates for transfer to oocytes at metaphase II. An additional advantage is that serum starvation for 5 days to induce quiescence is an easy treatment to apply, and it creates a stable population suitable for nuclear donors, which may be left sitting on the bench near to the microscope. This procedure seemed likely to be at least as effective as those for selecting cells in G1 phase, while also being reproducible and convenient.

Nuclei in G0 phase were transferred from the epithelial cell line to oocytes at metaphase II. The resulting embryos were cultured in temporary recipients for 7 days before being recovered and assessed. The effect of varying the exposure of the transferred nucleus to the oocyte cytoplasm was assessed by varying the time of activation in relation to nuclear transfer. Either nuclear transfer and activation were carried out at the same time, or activation was before or after nuclear transfer. However, this modification did not have a significant effect upon development of the reconstructed embryos, although the number of observations was very small. A total of five live lambs were born, of which two survived. These were the first mammalian offspring to be born following nuclear transfer from a cell that had been in culture for a prolonged period.

(c) The first adult clones

The following year, encouraged by our success, we set out to conduct a classical study of the effect of stage of differentiation of the nuclear donor upon developmental potential after nuclear transfer. A new cell line was derived from the
embryonic disc of a day 9 blastocyst according to the protocol that had been used the previous year. A line was also established from an eviscerated day 26 fetus. Our collaborators, PPL Therapeutics had a cell line in culture derived from the mammary gland of a 6-year-old ewe in the last trimester of gestation, established originally for studies of milk protein gene expression. These cell lines were all from ewes of different breeds. Furthermore, none of them were Scottish Blackface, which was the breed of all of the oocyte donors and recipient ewes. This enabled us to confirm the origin of lambs as soon as they were born on the basis of skin colour. Microsatellite analysis was also carried out to provide confirmation [27]. Live lambs were obtained from all three donor cell lines. An analysis was also carried out to provide confirmation [27].

Live lambs were obtained from all three donor cell lines. A significantly smaller proportion of reconstructed embryos reached morula or blastocyst stage (p < 0.001) if the donor nucleus was from the adult cells than fetal or embryo-derived cells (11.75 versus 27.4 and 39.0% for adult, fetal and embryo-derived, respectively). Christened ‘Dolly’ by one of the stockmen who cared for the sheep, the lamb derived from an adult mammary cell was the first clone of an adult animal born in July 1996. She was the subject of intense media and scientific scrutiny for her entire life. There were, however, two major limitations to detailed physiological studies. First, Dolly was the only adult clone and she was different from all other sheep in so many ways that there were no appropriate controls. She was of one genotype, but from an oocyte of a second breed, and her surrogate mother was from a third breed, and finally she was hand reared on milk substitute rather than being suckled by her surrogate mother. Any one of these factors could have influenced her growth and metabolism.

The report in 1998 that mice had been born following transfer of nuclei from adult cells by Wakayama and his colleagues in Hawaii [28] provided important independent confirmation that the procedure is applicable in a different species and reproducible. Cumulus cells from the ovarian follicle have a practical advantage that they are quiescent when recovered from the follicle. A number of differences in procedure between that used in sheep in Roslin and the mouse work in Hawaii were essential for their success. First, mouse oocytes and zygotes are fragile in comparison to many other species requiring different micromanipulation. The Yanagimachi laboratory had great experience in manipulation of mouse oocytes, particularly their pioneering use of piezo-driven micromanipulators for intracytoplasmic sperm injection (ICSI) as used in studies of gametogenesis and fertilization [29]. Later they were to recommend to groups wishing to learn their nuclear transfer procedure in mice to rehearse procedures for ICSI first before attempting nuclear transfer.

In addition to this technical difference, they reported an aspect in the protocol that was essential for normal development to term after nuclear transfer. When oocytes were activated at the time of nuclear transfer many of the embryos developed to the blastocyst stage, but none developed to term [28]. By contrast, if oocyte activation was delayed for between 1 and 3 h after injection of the nucleus, a greater proportion of embryos developed to blastocyst and live offspring were obtained [28]. Other research showed that delayed activation offers an advantage in cattle [30], but has no effect in sheep [26]. The molecular basis of this effect is not known.

Although Dolly was a healthy and fertile animal, it was discovered early in her life that her telomeres were short in contrast to those of age-matched controls. An analysis of telomere length at 1 year of age showed that telomere length was reduced in all three cloned lambs studied, one lamb obtained from each of embryo-derived, fetal and adult cells in the experiment, but the effect was greatest in Dolly, derived from an adult cell. However, the extent of telomere shortening was more strongly associated with the period of cell culture, than the developmental stage of the donor cell [31]. The unusually short length of the telomeres in Dolly was confirmed in an analysis after her death (S Rhind, W Cui, T King, D Wylie, I Wilmot 2003, unpublished observations). An experiment over a period of up to 20 years involving tens of animals produced by both natural mating and nuclear transfer, would be required to discover whether the shortening had any physiological consequence. While normal farming practice does not allow this to happen, there are reports that the lifespan of sheep can be up to 20 years [32]. There is one other case of telomere shortening in cloned animals, but in other studies this effect has not been observed. In a later detailed study in cloned sheep at Roslin, telomere length was fully restored following nuclear transfer from fetal cells [33].

In most other regards, Dolly was a healthy, fertile animal. She produced a total of six live lambs before it was decided that she should not be mated again. At that time, it was thought that she might live for a total of 20 years, and it was felt that to produce lambs every year for that period was not acceptable treatment of an experimental animal.

Dolly was euthanized on 14 February 2003 when examination confirmed that she had a large tumour in her lungs. A detailed histopathology was carried out following her death and the only abnormality that was identified in addition to the lung tumour was arthritis in a hind leg. It was confirmed that the tumour was due to a viral infection. There is no treatment for this progressive condition, which ultimately restricts the ability of the animal to breathe. It was kinder to end her life at that point rather than allow her condition to deteriorate. There is no reason to think that her being a clone contributed to the development of the tumour; the infection was present on the farm. The virus has a very long incubation period when it cannot be detected, which makes its elimination very difficult. Dolly was housed for her own security and keeping animals in close proximity increases the risk that the infection will spread.

Institute veterinarians had recognized and treated the arthritic condition 2 years previously. She was the only clone in which arthritis occurred and it may have arisen because she was treated differently because of her unique significance in biology. Over the years, she was photographed hundreds of times and we got into the habit of using food to encourage her to come to a specific point for a photographer. As a result, she became overweight until a diet was introduced. Secondly, she often stood on her hind legs against a fence in a way which is unusual for sheep, which would have placed an unusual load upon her joints. Thirdly, she was housed indoors on hard floors for most of her life (for security reasons) and this may have stressed her joints. We cannot know whether or not being a clone made her more vulnerable to arthritis, but the condition is not common among cloned animals, either at Roslin or elsewhere.

(d) Cloning in other species

Announcement of the birth of Dolly stimulated research in many mammalian species and in most cases offspring were produced (see [15]).
However, there were species in which cloning was not reproducible, including particularly the rat and primates in general.

Rat oocytes have been found to activate very readily and so are difficult to maintain in metaphase II. In one study, 40% of oocytes had activated, albeit abortively, within 1 h of recovery from the oviduct [34]. The authors reasoned that activation reflected the loss of MPF activity by a proteasome-mediated pathway and introduced a proteasome inhibitor, MG132, which had been shown to block the meiotic transition from metaphase to anaphase in the rat [35]. They noted that this stabilized most (77%) metaphase II oocytes for up to 3 h [34]. In a modification of the Wakayama protocol, nuclear transfer in the rat was performed by transfer of nuclei in mitosis into oocytes at metaphase II. The insertion of the donor spindle and then removal of the metaphase II oocyte were performed sequentially with one introduction of the piezo-driven pipette. Two viable offspring were obtained after transfer of 129 embryos derived in this way into recipient females. These observations demonstrated that cloning of rats is possible, provided that the recipient oocytes are stabilized in metaphase II.

(e) Primates

In an early experiment in non-human primates, viable offspring were obtained when nuclei from early cleavage stage embryos were transferred [36]. Two cloned rhesus monkeys were born following transfer of nuclei from 4- to 8-cell stage embryos (2 live born from 22 embryos transferred to recipients), but none following transfer from 8- to 16-cell stage embryos (0 from 20 embryos transferred). Early research suggested that this failure was caused by removal of key factors from the oocyte during enucleation. Inspection of recipient rhesus monkey oocytes revealed ‘disarrayed mitotic spindles with misaligned chromosomes’ [36]. Many of these embryos cleaved to yield aneuploid cells. By contrast, when nuclei were transferred into fertilized embryos many of the reconstructions were successful as judged by formation of interphase nuclei and appropriate alignment of chromosomes on bipolar spindles [36]. In a later experiment, somatic human nuclei were transferred into human oocytes which were either intact or had been enucleated [37]. Whereas blastocysts formed and triploid ES cells were derived from them if the recipient oocyte was intact, if the oocyte had been enucleated development ceased at late cleavage stages at the stage when transcription from the embryonic genome is usually initiated. Taken together all of these observations suggested that primate oocytes are at least not well suited for other factors upon development of cloned human embryos [40]. Specifically, it was noted that exposure to high concentrations of Sendai virus to induce cell fusion also induced an increase in intracellular calcium concentration that was able to induce activation. In later experiments, the Sendai virus preparation was diluted up to 20-fold. In addition, in some experiments, calcium was omitted from the medium until the stage at which the aim was to induce activation. Taken together the observations in these two pioneering laboratories suggest that the barrier to SCNT in primates has been overcome. In principle, it is now possible to use SCNT to generate patient-specific ES cells for use in cell or gene therapy.

(f) Limitations of the nuclear transfer procedure

Although the procedure established at Roslin has proved to be effective in many different species in laboratories around the world, it has several limitations. It remains an inefficient procedure with only a small proportion of reconstructed embryos developing to term and a greater than normal postnatal loss [41]. In this detailed study in cattle, prenatal loss occurred throughout gestation, although the greatest proportion was within the first 50 days. There were a variety of different causes of death. Far greater than normal prenatal loss is reported in all species although the precise cause varies.

In addition to death of cloned offspring, there is an increased incidence of abnormalities in surviving offspring. An increase in birthweight is the most common perturbation. The nature of the change was revealed first in an analysis of the use of the cloning procedure developed by Willadsen in...
cattle, while he was working at College Station in Texas for Granada, a cattle breeding company [42]. In a very well-designed study the authors monitored birth weight and growth in calves resulting from a number of different types of mating. In one control group, females were either naturally mated or inseminated with semen from a group of bulls. These same bulls were used to inseminate females that had been induced to superovulate in order to provide an increased number of embryos for transfer into recipient females. These procedures created groups of full and half-sibs. Finally, in the third group nuclei from some embryos recovered from the donors were used to provide nuclei for transfer to produce cloned offspring so adding further groups of full and half-sibs along with groups that were genetically identical. After adjusting for sire or dam effects where appropriate, the calves produced by nuclear transfer were approximately 20% heavier than those derived either by mating or embryo transfer. More striking still, the variability in birth weight of clones was 4–12 times greater than that for the other groups [42]. In other words, the groups that were expected to be very similar were in fact less uniform. The differences in weight had almost completely disappeared by 1 year of age. Increases in birth weight were also observed in several other experimental situations including embryo culture in the presence of serum (as reviewed in [43]).

Detailed analyses of the ‘large offspring syndrome’ revealed not only changes in birth weight, but also perturbation of coefficients of growth of major organs including liver, heart and kidney suggesting not only that overall growth was disturbed, but that development of major organs had been fundamentally disturbed [43]. These changes in sheep fetuses were found to be associated with changes in expression of \( \text{IGF2R} \), an imprinted gene [44]. Similar perturbation to development of cattle fetuses was found, and several groups noted a tendency to hypomethylation in the intergenic region between \( \text{H19} \) and \( \text{IGF2} \) genes [45]. In mice, the most obvious effect of nuclear transfer was on placental development, specifically in the spongiotrophoblast layer [46].

In reality, rather than being surprised that gene expression was sometimes abnormal in embryos produced by SCNT perhaps we should be surprised that viable offspring are ever produced. We ask an enormous amount of the recipient oocyte. It is disappointing to note that it is still the case almost 20 years after the birth of Dolly and over 50 years after the development of frogs following nuclear transfer from tadpoles that we still know very little of the mechanisms which bring about reprogramming following nuclear transfer.

**(g) Significance of the Dolly experiment**

The nuclear transfer research at the Roslin Institute was initiated with a view to being able to introduce precise genetic modifications in livestock species by making the desired change in cells that were then used as nuclear donors. During the 5 years before the cloning research was started, genes had been added to sheep by injecting several hundred copies of the transgene into a nucleus of an early embryo [47]. Although this technique was effective in producing ewes that made in their milk proteins that might be used to treat human disease, it was very ineffective. Only around 1% of injected embryos survived to become viable animals that made the specific protein in their milk and transmitted the transgene to their offspring. Most important of all, the technique was only able to add genes and could not make desired changes to the animals own genes. The Roslin procedure for nuclear transfer was used by others to introduce genetic change, for example, to create models of the human genetic disease cystic fibrosis in pigs [48]. However, a more effective alternative procedure for the introduction of precise genetic changes has been provided by the development of the ‘clustered, regularly interspaced, short palindromic repeat’ (CRISPR) technology described by Sander & Joung [49]. These systems are so efficient and accurate that it is possible to simultaneously modify several genes in mouse embryos using the CRISPR-Cas system [50], and with optimization this may also be possible in livestock species.

Despite these successes in biotechnology and research, there is little doubt that the greatest effect of the birth of Dolly was to make biologists think differently. Before the birth of Dolly, it was generally accepted that once a nucleus had assumed a specific differentiated state in an adult animal, it could not be changed to another. The birth of Dolly stimulated a number of groups to consider if they could find other ways of changing cells from one phenotype to another, most frequently to obtain pluripotent cells from populations of somatic cells. At the time of the birth of Dolly, nuclear transfer to produce cloned human embryos from which to establish patient-specific ES cells was one of the applications that was envisaged for the new SCNT procedure. However, an alternative method of producing patient-specific pluripotent stem cells has since been established independently by the laboratories of Yamanaka and Thomson. Cells which very closely resemble ES cells are obtained following the introduction of a small number of selected transcription factors into somatic cells such as skin fibroblasts [51,52] or peripheral blood cells [53]. The factors are known either to be essential for normal function of ES cells or are only expressed in ES cells.

It is now possible to produce induced pluripotent stem (iPS) cells in chemically defined media suitable for derivation of cells for clinical use [54]. There is then the question as to which approach to use for production of patient-specific pluripotent stem cells. It has been suggested that cells derived from embryos, albeit embryos derived by SCNT, might have fewer abnormalities than those derived by reprogramming. However, a recent study demonstrated a similar incidence of coding mutations and loss of imprinting in human pluripotent cells derived by nuclear transfer and the introduction of selected transcription factors [55]. A considerable research effort is directed now to the establishment of procedures for the derivation of iPS cells at clinical grade and their differentiation to tissue types that are required for cell therapy.

A significant contribution to research is already being made by use of iPS cells, and it is anticipated that in the future they will be used in cell therapy to provide the first effective treatment for many degenerative diseases. Such an outcome could not have been envisaged at the time of the first proposal by August Weissmann in 1885 for the use of nuclear transfer to answer a fundamental question of developmental biology [3].

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