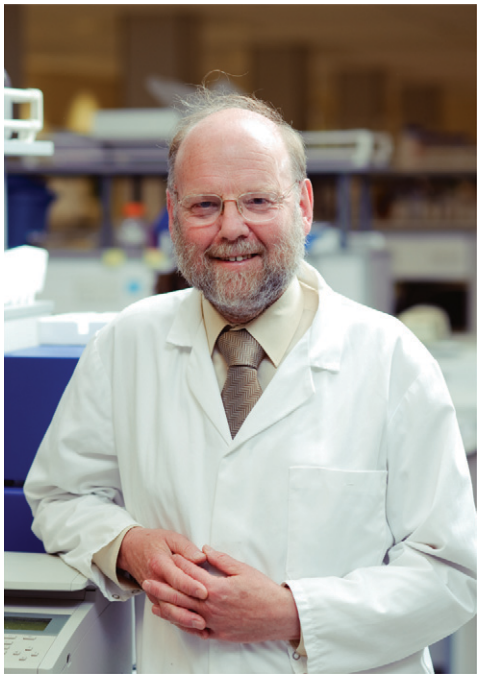

From Germ Cell Preservation to Regenerative Medicine: An Exciting Research Career in Biotechnology

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Abstract

Collection, manipulation, assessment, and storage of mammalian gametes, embryos, and stem cells are providing important opportunities in agriculture, research, and medicine. Semen and embryo freezing in livestock are used in breeding schemes, especially in cattle and for international trade, with no risk of spreading disease. In human medicine, they are used in treatment of infertility. Usually, knowledge gained in one species is applicable in the others. In one exception, some ruminant embryos cultured according to protocols used in human in vitro fertilization become unusually large offspring. This is due to disturbances in expression of imprinted genes. The nuclear transfer procedure developed at the Roslin Institute is being used to make genetic modifications in livestock to either direct production of biomedical proteins, create animal models of human disease, or enhance animal health and productivity. Human pluripotent cells are being used in Edinburgh to identify drugs to treat degenerative diseases.

INTRODUCTION

As I grew up I had no understanding of the nature of research and certainly no ambition to build a career in research. In part, this may have been because none of the cities in which I lived had universities at that time. I was born in a village between Coventry and Stratford-upon-Avon in 1944. Coventry was still recovering from a night of very heavy bombing on November 14, 1940. From Coventry, we moved to Saltaire, a suburb of the northern city of Bradford. Although both Bradford and Coventry have universities now, neither did at that time. When I was 14 we moved to Scarborough, on the east coast of Yorkshire, which was both a fishing harbor and a holiday resort. It was here that I first became interested in biology under the tutelage of a gifted teacher, Gordon Whalley.

Searching around for an occupation that was outdoors and could involve international travel, I began to consider agricultural extension work. With the help of family friends, I began to work on farms at the weekend and during vacations and enjoyed the work immensely. I applied for entry to the School of Agriculture at Nottingham University because it was one of the first to concentrate upon the science of agriculture, rather than on actual practice.

At that time, everyone did the same course during the first year before beginning to specialize; in my case, I selected animal science subjects. I was becoming more and more interested in laboratory work and relished the fact that we were approaching the stage of learning when there was some uncertainty about the hypotheses that were being put forward in our lectures, which were the subject of intense discussion with some of the lecturers and postdocs.

At this time, I began to consider a career in research rather than extension work and wrote to laboratories seeking a position as an intern during my last summer vacation. The first person to offer me a place was Professor E.J.C. Polge, known to everyone as Chris, in the AFRC Unit of Reproductive Physiology and Biochemistry in Cambridge. This opportunity was to be one of the most important influences in my life.

AN INTERNSHIP

Chris Polge is best known as the person whose experiments led to the recognition that there are compounds, such as glycerol, that protect cells during freezing and thawing. It is well known that this discovery resulted from clear thinking following a mishap in storage of medium. At the time of the experiment, cells were being dehydrated by using concentrated sugar solutions. These solutions were kept in a cold room. One day, Chris noted far more motility of the frozen and thawed sperm and was unable to account for the dramatic transformation. In the end, a chemist was asked to discover what was in the unusual sample and identified polyhydric alcohol. A search for an explanation revealed that one of the other bottles in the cold room contained glycerol, and, in those days of sticky paper labels, it was suggested that labels had fallen off and been replaced incorrectly, leading to the confusion. It has always seemed to me that great credit was due to Chris for recognizing that there was something different in that tube, as it would have been all too easy to shrug his shoulders at the unexpected result and press on with what he was doing.

During my period as an intern, I was expected to wash glassware and help to look after the animals, but was able to join in the Unit discussions. I learned a great deal about the current research and about the workings of science. In those years before health and safety regulations, coffee was brought to the laboratory every morning, and everyone would pause for a chat about research and the major events of the time. Most important of all, I learned to identify and assess early mammalian embryos. Life in the lab was enlivened by the presence of Professor Billy N. Day, on sabbatical from the University of Missouri, Columbia, who has been a good friend ever since.

After my summer in Cambridge, I returned to Nottingham, determined to try to build a career in research. Thinking that his lab was full, I wrote to ask Chris Polge if he would write a reference for me if I applied elsewhere; it was my great good fortune to discover that he had a place, which he offered me.

DEEP FREEZE PRESERVATION OF BOAR SEMEN

My period as an intern was supported by the Pig Industry Development Authority, and it was they who provided a postgraduate scholarship, which was more generous than government scholarships in that it would offer more support to married students and their children. So later that summer, Vivienne and I were married, and two years later our first daughter, Helen, was born.

During the course of my project, I learned a great deal from three different sources. The first was my mentor, Chris Polge, who had a deep knowledge of reproductive biology, as well as of freezing and thawing of cells. He was immensely patient and tolerant of interruption. My experimental approach was revolutionized by a visit from Australia of Stephen Salamon of the University of New South Wales. Steve taught me about factorial experiments and the opportunity that they provide to examine the effects of several factors and any interaction between them. Initially I froze the sperm in ampoules, which are relatively cumbersome to use, but Steve showed me the pellet method of freezing. In this method, small indentations are made in the top of a block of solid carbon dioxide. This dry ice is at −79°C and evaporates away slowly. We made rows of small indentations along the top of the block and let fall small droplets of the diluted semen into each hole. At first, these droplets were shiny liquid, but they became dull as they froze, and at that point they were tipped into a filter funnel that led into a test tube sitting in liquid nitrogen (−196°C).With a little practice, it was possible for one person to conduct a replicated multifactorial experiment in two days by diluting and freezing the sperm on day one and thawing and assessing the coded samples on the second day. The community of cryobiologists is comparatively small, so it was possible for even a student to know the leading lights, such as John Farrant and David Pegg of the Medical Research Council Clinical Science Centre and Stan Leibo and Peter Mazur of Oak Ridge National Laboratory, Tennessee. I learned a great deal from the small national meetings of the Society for Low Temperature Biology.

I was fortunate in beginning my research after a great deal had been learned of the factors that influence the response to freezing. In particular, it was clear that survival of cells was influenced by type of cell, rate of cooling, rate of warming, and choice and concentration of protective agent. If the rate of cooling is fixed, as is the case with pellet freezing, then it is necessary to vary the other factors. Soon after the discovery of the protective effect of glycerol, Chris Polge and James Lovelock (who is now best known for his Gaia hypothesis) carried out an analysis of the factors that influenced survival, most particularly the protective mechanism of glycerol. The fact that for each cell type there is an intermediate optimum rate of cooling suggests that at least two factors contribute to cell death. It is believed that cooling too quickly leads to formation of ice within the cells, whereas if cooling too slowly the cells suffer lethal exposure to concentrated salt solution in the remaining liquid. The effect of glycerol is to lower the temperature at which specific concentrations of salt arise and so reduce the damage caused (1).

The most important observation in my project was of the harmful effect of glycerol upon the acrosome of boar sperm. This new understanding arose from a comparison of the fertilizing ability of freshly recovered sperm, frozen and thawed sperm, and sperm held in diluent but not frozen and thawed. Whereas approximately 30% of the frozen and thawed sperm were motile, only 5% had a normal fertilizing ability when deposited surgically at the top of the uterine horns of a female pig. It was suggested that this difference might reflect damage to the cell membrane, reducing the

sperm's ability to pass from the uterus into the oviduct. In a series of experiments, the effect upon acrosome morphology was measured along with an estimate of motility of the frozen and thawed sperm. Whereas maximum motility was obtained with 4% glycerol, this concentration caused damage to the acrosome. In a final experiment, frozen and thawed sperm were deposited in the uterotubal junction, and the proportion of eggs fertilized was greatest with 2% glycerol when compared with 0%, 1%, 3%, 4%, and 5% glycerol (2). Other laboratories came to similar conclusions, and the use of lower concentrations of protective agent in order to avoid damage to the acrosome is still in use today.

THE FIRST CALVES FROM FROZEN EMBRYOS

I was very fortunate to be offered a postdoctoral position with Chris Polge to study the possibility of storing cattle embryos recovered surgically from superovulated donors. This project arose because of a very exciting report describing the storage of mouse embryos at −79°C (3). Although this result has never been replicated, it generated a great deal of research to find an effective procedure. I applied the knowledge that I had gained freezing semen in an empirical study of the effect of rate of cooling and warming on embryo survival. The expectation was that the optimum cooling rate would have to be very slow (less than 1°C/min), because the cells of early embryos are very large. Optimum cooling rate depends upon the amount of water in the cells and the ease with which it can leave the cell (4), and a systematic comparison was made of the effect of cooling rate, warming rate, and the use of different protective agents.

To compare treatments with embryos recovered from each superovulated donor, we copied the approach used by John Farrant and his colleagues at Northwick Park. We set up an apparatus with four large vacuum flasks, which at the appropriate time were filled with liquid nitrogen. Into these were lowered a variety of different containers, including a smaller vacuum flask, a double-walled container that had not been evacuated, and an ordinary laboratory beaker (**Figure 1**). In some cases these were filled with ethanol, but in others they were filled with air. In this way, we were able to cool the embryos at reproducible rates ranging from 0.2°C to several hundred degrees centigrade per minute when measured between −10°C and −70°C. Whereas optimum survival of most mammalian cells is achieved with maximum rate of warming, in the case of embryos it was found to depend upon slower warming. These rates were achieved through essentially the same approach, namely by insulating the frozen embryos from the warm environment in different ways.

As assessed in culture, survival of mouse embryos was obtained with permeating protective agents, e.g., 1.5 M dimethyl sulfoxide, provided they were cooled very slowly (0.2°C/min) and warmed slowly (approximately 10°C/min). This is in marked contrast to boar semen, which was cooled at approximately 100°C/min and warmed even more quickly. Another difference was in the procedure for removal of the cryoprotective agent. It was believed that the cells suffered an osmotic shock if embryos were transferred directly to culture medium because water could enter the cells very much more quickly than the agent could leave. It was necessary either to remove it by dilution in a series of steps or to include a nonpermeating sugar in the medium to shrink the cells. The presence of the sugar reduces the total amount of dimethyl sulfoxide in the embryo and so reduces the shock when water rushes into the cells. The first calf was produced in June 1973, and a second was produced later in the year after late blastocysts were frozen and thawed according to this protocol.

Research to preserve earlier stages of development revealed that embryos of some species are sensitive to cooling above freezing point. As opposed to sperm sensitivity to rate of cooling, this is sensitivity to lower temperatures. Mouse embryos at all stages were tolerant of chilling, whereas

Figure 1

The apparatus used to obtain different rates of cooling cells.

sheep and cow embryos were killed by cooling in ice until the morula/blastocyst stage, and pig embryos became tolerant of cooling only at the late-blastocyst stage.

PHYSIOLOGICAL VARIATION IS A MAJOR CAUSE OF PRENATAL MORTALITY

After completing the research on embryos, I was offered a position in the Animal Breeding Research Organization in Edinburgh. This institute, which has metamorphosed to the Roslin Institute over the intervening period, was concerned with genetics of farm mammals, and my role was to make available methods for assisted reproduction in animal breeding. To do this, handling facilities for cattle and pigs needed to be built, and this took several years. In the meantime, I began to investigate causes of prenatal mortality in sheep. This research arose from curiosity about the need to control the relative stage of reproductive cycles of donor and recipient females, if embryos are transferred between two sheep or two cattle. Typically, "time" is measured as the interval after the onset of estrus. Pregnancy will very rarely occur if there is a three-day difference between the two females, regardless which animal is in estrus first, but in sheep and cattle some pregnancies will be successful if there is a two-day asynchrony in either direction. By contrast, there is an asymmetrical relationship in pigs in that it seems that the embryo can be in advance of the recipient but is less tolerant of being delayed in development.

A critical experiment arose from discussions with David Sales, a mathematician in the Institute. We hypothesized that in sheep there are changes in uterine secretion and that normal development

depends upon the embryo being at an appropriate stage of development to respond to these changes in secretion. In search of these effects, we transferred embryos into an advanced recipient for three days (**Figure 2**). These embryos were then recovered and transferred to a second recipient at the same stage as the original donor female. We began this experimental treatment when the embryos were either day 3 or day 6. Control embryos recovered at these two stages were transferred twice, both times into recipients that were in estrus at the same time as the original donors. These comparisons enabled us to identify the effects of being in an advanced uterine environment as day-3 or day-6 embryos, when the embryos were 8-cell and morulae or early blastocysts, respectively.

We found that the effect of asynchrony was different at the two stages of development. Embryos transferred as day-3 8-cell embryos were only slightly affected by the advanced environment, whereas those transferred as day-6 morulae or blastocysts were markedly advanced in their stage of development. These observations confirmed that changes in uterine secretions can influence development of early mammalian embryos.

The subsequent development of the embryos was assessed by recovering any fetuses on approximately day 35 of pregnancy, and again there was a difference in the effect of the advanced environment at the two stages of development. There was no effect upon survival if the first transfer was as day-3 embryos, but the fetuses were significantly larger. By contrast, those first transferred as day-6 embryos were significantly less likely to have survived.

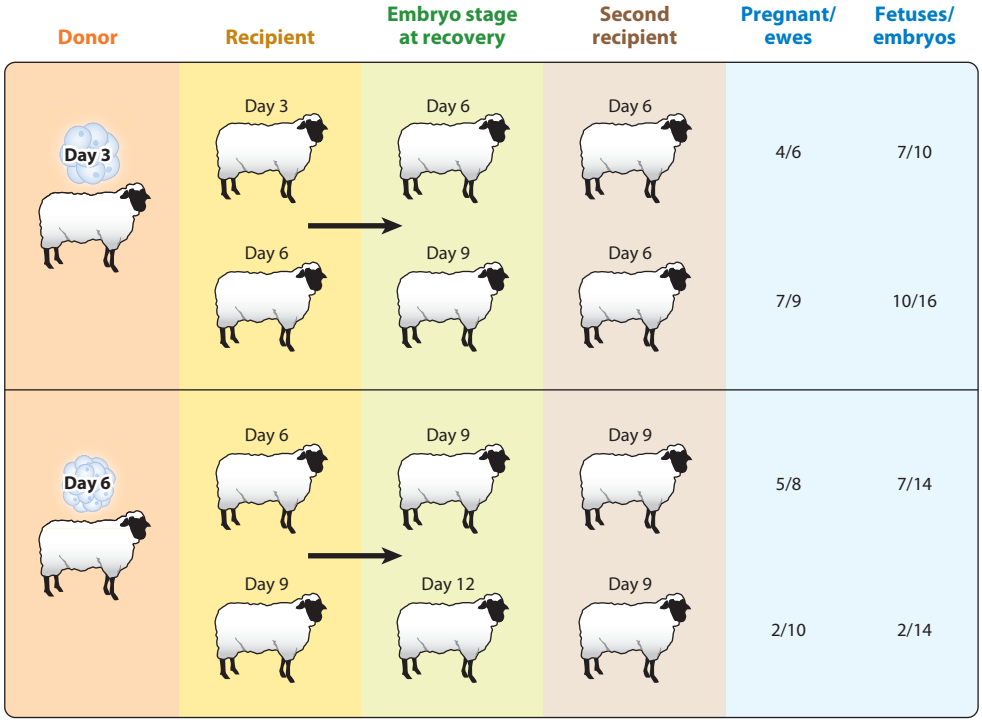

Figure 2

Sheep embryo transfer experiment design. This experiment tested the hypothesis that asynchronous embryo transfer leads to embryo death because embryos become abnormal if exposed to an inappropriate uterine environment at specific stages of development. Embryos were recovered from donors on either day 3 or day 6. To assess the effect of an asynchronous environment, some were transferred to ewes three days in advance of the donor. After three days, they were recovered and transferred to second recipients synchronous with the original donor. Asynchronous transfer at day 3 (8-cell embryos) did not affect survival, whereas asynchronous transfer at day 6 (morulae and early blastocysts) caused a significant reduction in survival in the second recipient.

These observations confirmed that the relationship between uterine environment and the embryo was critical for normal development and survival during this period, days 6–9 of development.

Earlier experiments by Neil Moore and his colleagues in Sydney had shown that endocrine control of uterine function occurred in several stages. They had systematically supplied hormones via daily injection to ovariectomized ewes before transferring embryos to these ewes to assess uterine function (5). There was a need for priming with progesterone to mimic a previous luteal phase, estradiol equivalent to that at estrous, and then an increasing dose of progesterone to mimic the changes seen after ovulation. In a subsequent study, they assessed the effect of varying the time of the rise of progesterone in relation to the estradiol and found that, as judged by embryo transfer, the time of changes in uterine function was determined by the time of the rise in progesterone (6).

Even in healthy, well-managed livestock, some 20% of eggs that are fertilized fail to become live offspring at birth. A majority of this loss occurs within the first trimester of pregnancy, and the causes are not well understood. Taking the results of these two studies together, it seemed possible that at least some of this loss might reflect physiological variation in the mechanisms that regulate early development and the establishment of pregnancy. The pattern of the rise in progesterone level certainly is highly variable, as is the stage of embryo development at any particular time after estrous is detected; however, it may be that this reflects variation between animals and that uterus and embryo are usually in perfect synchrony. In one study to examine the effect of variation within females (7), embryos were recovered from 19 sows on days 4–8 before the embryos had been distributed around the entire uterus. Ligatures were placed approximately 25 cm below the tip of each uterine horn, and that distal region was flushed for embryo recovery. The lower region of each horn was then divided in two by a ligature, and further ligatures were tied at the bifurcation of the uterus. In this way, four unflushed regions of the uterus were established. The embryos were divided into four groups based on their apparent stage of development, and each group was transferred to a segment of the uterus of the animal from which they had been recovered. When recovered before there was a risk of luteolysis caused by a segment of uterus that had no embryos, the proportions of embryos surviving were 67%, 49%, 55%, and 31% (SE of means 8%) in the four groups judged to be most advanced to least well developed, respectively ($p < 0.05$). These observations suggest that, in pigs, there is sufficient variation in stage of embryo development to prejudice embryo survival.

A detailed consideration of factors that could lead to prenatal loss because of physiological variation has been presented elsewhere (7), and it still seems to me that the basic hypothesis that some prenatal mortality is caused by physiological variation in embryo development or maternal control of uterine function can account for many observations on mammalian reproduction. Factors that may influence embryo survival in this way are shown schematically in **Figure 3**. It is frustrating that it is impracticable to test the hypothesis. To assess whether a specific factor causes prenatal loss, an experiment is required in which the factor is eliminated while there is no change in any other factors. This in itself is almost impossible to achieve. To detect differences in survival of 5%, which might be the loss associated with any one factor, groups of more than 100 females per treatment are required. On both biological and economic grounds, it seems very unlikely that trials of this type are possible.

BIOMEDICAL HUMAN PROTEINS PRODUCED IN THE MILK OF LIVESTOCK

In 1985, decisions were taken to introduce molecular biology into the Institute and to do this by establishing a transgenic animal project. The very skilled team that I had assembled and trained

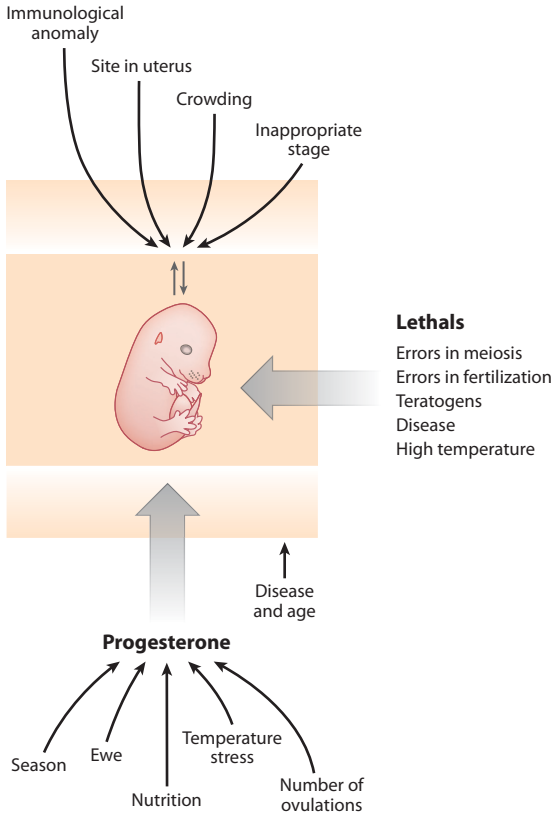

Figure 3

Factors associated with prenatal loss, particularly in sheep, are shown according to the route by which they act. Those at the top of the diagram disrupt the relationship between embryo and mother, those on the right cause abnormal embryos, and those at the bottom of the diagram act through the maternal environment [see Wilmut et al. (7) for references]. Adapted from Reference 7 with permission from the publisher.

was required for this project. After some consideration, the decision was taken to produce in the milk of farm animals human protein needed to treat a human disease. Although cattle were expected to be the ultimate production animals, they are very expensive and have a generation interval of two years. By contrast, sheep are much cheaper and have a generation interval of only a year, and we had a great deal of experience in recovering and transferring their embryos. A third choice, the rabbit, was also considered but was never used. At this point, there was one publication describing gene transfer in livestock (8), and the authors had concentrated upon modification of growth in pigs. There was relatively little research on sheep. The project was led by Professor John Bishop of the Department of Genetics of the University of Edinburgh and John Clark of the Animal Breeding Research Organization, who was a former colleague of Bishop's.

The basis of the project was to modify sheep genetically by injecting a few hundred copies of a gene into an early nucleus. In a small proportion of cases, copies of the gene were integrated into the chromosomes of the resulting offspring. We recruited Paul Simons, who had just completed a PhD with Ed Southern in Edinburgh that involved injecting into cells. As cells are much smaller than embryos, we were sure that he would master quickly the art of locating the nucleus in early embryos and then injecting into them. A practical difficulty with micromanipulation of livestock

embryos is that they are opaque because of lipid-filled vesicles in the cytoplasm. Even with good-quality Nomarski optics, it was difficult to see the pronuclei. However, Paul soon acquired the knack after he was shown pronuclei in fixed oocytes.

During the next few years, we demonstrated in turn that the team could transfer genes (9) and that the transgenes sometimes functioned appropriately and were transmitted to their offspring, and we finally obtained human protein in the milk of transgenic sheep. The basis of the project was to use the regulatory elements of sheep β-lactoglobulin with the coding sequences of the human protein as a cDNA. The suitability of this construct was assessed in transgenic mice as a proof of concept before the transgenes were introduced into sheep.

This was also the era in which commercialization of research became a priority, whereas in the past it had been discouraged. Production of biomedical proteins was a target that could gain the support of investors. A company was formed on the basis of the technology, and it became both a scientific partner and a financial supporter of our research. We became familiar with the need for confidentiality and to negotiate for the use of commercially protected constructs. We came to the point for technology transfer when the company had its own veterinary and embryology teams. We planned two weeks of embryo manipulation; the Institute took the lead in the first week and demonstrated the techniques that we used, and the company team carried out the procedures in the second week, with us in attendance in case our assistance was needed. The process worked perfectly, and it was from this work that the first lambs were born that could produce commercially useful human protein (10). The choice of α_1-antitrypsin (AAT) was influenced by several factors. The gene is small, whereas those encoding clotting factors that were used in other studies are unusually large and complex. AAT was available for commercial use, and there was the potential to offer the protein for treatment of not only emphysema but also cystic fibrosis.

The opportunity to take part in this project changed the direction of my research. It brought home to me that gene transfer is inefficient, as very few injected embryos (only approximately 1%) become viable offspring in which the transgene is expressed, but even more importantly, it is possible to add only a single gene. It was around this time that biologists were really beginning to reap the benefits of being able to introduce precise changes into mice through the use of embryonic stem (ES) cells. This technology still continues to provide extraordinary opportunities in research. At a meeting in 1987, I learned during a conversation in a bar that Steen Willadsen, the pioneer of nuclear transfer in mammals, had produced calves by nuclear transfer from blastocysts. As this is the stage of development from which mouse ES cells are derived (11), I speculated that if we could derive ES cells from livestock, we would be able to derive offspring by nuclear transfer, so avoiding the chimeric generation, which would be a considerable roadblock in animals with a long generation time. I felt that we might be able to use this combination of techniques to introduce precise genetic change into livestock for the first time. It was with this possibility in mind that we sought funds for a collaborative project to derive ES cells from cattle and develop a method of nuclear transfer from them. We were building upon a project by Lawrence Smith, who came to the Institute in 1984 with a very strong ambition to study nuclear transfer.

DEVELOPMENT OF A NOVEL PROCEDURE FOR SOMATIC CELL NUCLEAR TRANSFER IN SHEEP

The subject of nuclear transfer was controversial at that time because of the work of Illmensee (12), which had proved to be unrepeatable. As part of an analysis of these claims, James McGrath & Davor Solter (13) established a repeatable procedure for nuclear transfer between zygotes and then carried out a detailed analysis of development of mouse embryos produced by transferring nuclei from later blastomeres to enucleated zygotes (14). Whereas transfer between zygotes did not affect

subsequent development in vitro, fewer embryos developed after transfer from 2-cell embryos and none at all from 4- or 8-cell embryos (14, p. 1319). This clear result and awareness of the recently defined effects of imprinting prompted the authors to speculate that "the simple nuclear transfer of mammals is impossible." This comment came back to haunt them a few years later.

Progress in nuclear transfer depended upon many technical innovations that went unnoticed except by those of us who were involved in using the technique. In research in which they used enucleated zygotes as the recipient cell, McGrath & Solter (13) employed inactivated Sendai virus to introduce the transferred nucleus by fusing the karyoplast to the recipient oocyte. In his work with sheep, Willadsen (15) used oocytes that had been recovered soon after ovulation, at which time they would have been at the second metaphase of meiosis. However, with hindsight we can see that he then held them for so long that many probably activated spontaneously in response to an environmental stimulus. He obtained enucleated fragments by splitting the oocytes in two when he was able to reason that in all probability there was no pronucleus in one fragment. Tsunoda and colleagues (16) used a DNA-specific fluorochrome to visualize the second metaphase and showed that this did not cause harm to the cytoplasm, provided that the exposure to UV occurred for less than 15 s. As the impression gained strength that the oocyte at metaphase II was a more effective recipient, as was confirmed later (17), a variety of different protocols were developed for the parthenogenetic activation of the reconstructed oocyte. The methods that were in common use varied between species. Exposure to ethanol or strontium was common in the mouse, whereas in livestock electrical pulses were in general use (18).

Lawrence Smith was the first person in the Roslin Institute to investigate methods of nuclear transfer in mouse and sheep in a postgraduate project. Soon after the publications by McGrath and Solter, he extended their studies by examining the effect of cell-cycle stage on development of mouse embryos reconstructed by nuclear transfer. He first transferred into enucleated zygotes nuclei from other zygotes either early or late in the cycle or nuclei from 2-cell embryos either early or late in that cycle and then compared the effect of transfer to the same or different stage of the cycle (19). When transferring between zygotes, development to blastocyst in culture was highest when transfer was synchronous (94% versus 76%). By contrast, when transferring from 2-cell embryos, development to blastocyst was greatest if both donor nuclei from 2-cell embryos and zygote-derived cytoplast were late in their cycle.

There was some concern at this stage in the development of the technology that the changes associated with the onset of transcription from the embryonic genome would present a barrier to normal development after nuclear transfer. To assess this possibility, Smith transferred nuclei from sheep 16-cell embryos and the inner cell mass of day-6 blastocysts into oocytes enucleated at metaphase II of meiosis (20), stages when the embryonic genome is fully activated. Several technical refinements were made in this study. For example, electrofusion rates were higher if an alternating current was applied before the pulse of direct current that was used to fuse the cells (88% versus 47%) and when the larger cells from 16-cell embryos were being fused (82% versus 47%). The addition of cytochalasin B to the culture medium directly after fusion enhanced development of embryos (11% versus 35% with 16-cell stage embryos and 0% versus 56% with inner–cell mass cells). This outcome suggested that mechanisms that involve actin microfilaments influence early events after nuclear transfer. Of the 23 embryos that reached morula or blastocyst stage and were transferred to recipients, 4 developed to term (17.4%). As transcription is initiated at the 8–16-cell stages (21), these observations strongly suggested that it is possible to obtain development after nuclear transfer of nuclei in which transcription is taking place.

The next analysis was of the effect of varying cell-cycle stage of both donor cell and recipient oocyte when transferring 8-cell mouse nuclei into activated oocytes at different stages of the first cell cycle (22). This project was carried out by Pedro Otaegui, who returned to Barcelona after

completing his project at the Institute. The first requirement was for cells at the desired stages of the cell cycle. Unfortunately, early cleavage-stage embryo cells do not respond to the usual checkpoints. To obtain cells from 8-cell embryos, it was necessary to hold 4-cell embryos in mitosis with nocodazole and, after releasing the cells, to observe how long it took to reach the different phases of the cycle. It was also necessary to prove that the concentration of nocodazole that was used and the duration of exposure did not have adverse effects upon subsequent developmental potential. Only then was it possible to attempt the nuclear transfer and assess development to blastocyst.

Development of cloned embryos was influenced by the cell-cycle stage of both donor nucleus and recipient oocyte. Factors that were expected to influence development were the occurrence of inappropriate chromosome condensation in an oocyte at metaphase II and the ability of the cytoplast to reprogram gene expression, about which less is known. By contrast, it was not expected that oocytes in S-phase would cause premature chromosome condensation. The highest proportion of embryos developed to blastocyst when nuclei in G1 were transferred into oocytes in metaphase II or early S-phase (43% and 60%, respectively NSD). This study emphasized the importance of coordinating cell cycle in reconstructed embryos and gave some understanding of promising approaches (**Table 1**).

THE FIRST CLONE OF AN ADULT ANIMAL

The molecular mechanisms that regulate cell cycle in embryos were analyzed in a series of critical experiments led by Keith Campbell, and in a few years this work provided the first protocol to clone adult animals. In his thesis work at the University of Sussex, Keith had tried to isolate and characterize maturation/meiosis-promoting factor (MPF). As a result, he was very familiar with the role of this enzyme in the regulation of cell cycle and with methods for assessing its activity. In the first study, he chose to use bovine oocytes because they were available from slaughterhouse material in large numbers at all times of the year, unlike those of sheep. As measured by H1 kinase activity, MPF level declined immediately after activation of the oocyte, to approximately 30 + 4% of the maximum level by 60 min postactivation and to 20% after 120 min (23).

A comparison was then made of the effect of transferring nuclei into oocytes with maximal MPF activity or 10 h later when MPF could not be detected (23). Regardless of the stage of the cycle

Table 1 Effect of cell-cycle stage of donor mouse 4-cell blastomeres and recipient oocyte on development to blastocyst in culture[a]

Cell-cycle stage of oocyte	Cell-cycle stage of nuclear donor		
	G1	S	Late-S
Metaphase II	60 43%	55 0%	60 0%
Early S	50 60%	46 14%	61 0%
Late-S–G2	56 0%	53 0%	57 12%

[a]For each treatment combination, the number of reconstructed embryos is shown along with the percentage that reached the blastocyst stage. Each treatment was replicated at least three times.

of the nuclear donor, when nuclei were introduced at the time of activation in the presence of a high level of MPF, this was followed by nuclear membrane breakdown, chromosome condensation, and DNA replication. By contrast, the nucleus determined the response following transfer 1 h after activation. Those in G1/S underwent DNA replication, whereas no replication was observed in nuclei in G2 phase. This led us to develop two different approaches to nuclear transfer. In the first approach, the oocytes were activated to induce decay of MPF activity, in which case the nucleus determined whether or not DNA replication occurred. We called this the Universal Recipient because it was expected to lead to normal ploidy in all cases. The second approach was to transfer nuclei into oocytes at metaphase II, in which case the nuclei had to be in G1 phase awaiting DNA replication. This approach is difficult to employ because it is impossible to synchronize blastomeres in G1.

The potential of the Universal Recipient was assessed by transferring nuclei from unsynchronized 16-cell embryos to either oocytes in metaphase II or activated oocytes in S-phase. There was a significant difference in development to blastocyst stage (21.3% versus 5.4%, respectively). Normal lambs were born following transfer of blastocyst produced by both protocols (24). In hindsight, many of the early nuclear transfers in livestock may have used a preactivated recipient oocyte either inadvertently or deliberately (e.g., Reference 15).

The long-term objective of the projects that were funded at the Roslin Institute was the ability to introduce precise genetic change by genetic modification of cells in culture before using them as nuclear donors. At that time, there were no ES cell lines from livestock embryos, but our colleague Jim McWhir had observed cells with some of the characteristics of ES cells in cultures of sheep embryos. He reported that they simply disappeared during prolonged culture. They were apparently not lost by differentiation into other cell types. We designed an experiment to carry out nuclear transfer from cells taken at different intervals after the start of culture, with the expectation that some cells in early cultures would still support normal development to term, whereas this ability would be lost at the critical stage in the cultures as differentiation occurred. Whereas offspring were obtained following nuclear transfer from cells at early passage in culture, none were obtained at later stages of the culture. This seemed to agree with the earlier experience that nuclear transfer from differentiated cells would be impossible. The number of nuclear transfers carried out was too small for us to draw clear conclusions on this point; however, we continued with the research, and a significant innovation was made in the process of carrying out the study. Keith came to see me to point out that the cell cultures that we were using would probably respond to serum starvation by becoming quiescent, and this proved to be the case. For the first time, we had a convenient and accurate method of obtaining cells in G0/G1—a very different situation from that in which Pedro Otaegui and several others around the world had worked. Nuclear transfer was carried out in cells that were rendered quiescent by serum starvation, and as a result, we obtained the first live offspring following nuclear transfer from a cultured cell line (25). There is no doubt in my mind that our success was due to the use of quiescent cells, but since then others have shown that cells in early G1 are also effective. However, these cells were selected by frequently observing cells in culture and selecting cells within minutes of cleavage, which is much more time consuming than simple serum starvation (26).

The successful outcome of the experiment depended upon learning about the disturbance to parturition in animals carrying cloned fetuses. We never had a lamb born at or before the date that would be expected for the breed of lamb. Aware of the tendency for a delay in parturition, we adopted a strategy of inducing birth soon after the normal date using well-established schedules for the administration of corticosteroid. Several lambs died around the time of birth despite appearing to be fully developed. We were then advised by a very experienced large-animal veterinarian to leave the ewes to their own devices and to intervene only if a ewe

became distressed. After this we had fewer problems at birth, and many more lambs survived and thrived.

In light of this success, we began to plan the next experiment. In view of the well-known demonstrations that success rate declines as nuclei are taken from later stages of development in amphibians and mammals, we considered carrying out nuclear transfer from embryo-, fetal-, and adult-derived cells. However, our ambitions were limited by the funds available from the Ministry of Agriculture, Fisheries and Food. We were fortunate in that PPL Therapeutics was interested in the potential use of cells cultured from embryos for genetic modification and offered to fund a repetition of the work with cells derived from late blastocysts. Our government funds could then be used to transfer nuclei from fetuses as the logical next step. At that stage, it seemed that our funds would not allow us to transfer nuclei from adult cells. In Edinburgh, the sheep breeding season runs from October to March, and at the beginning of the New Year we reviewed what had been achieved in the first half of the season, as well as our plans for the remainder.

By that time, we had derived blastocysts from both embryo- and fetal-derived cells. Meanwhile, PPL Therapeutics had found it difficult to replicate the derivation of the embryo-derived cells, so that some of the sheep that had been purchased were not required to complete their contract. Because they were as curious as Keith and I to discover if we could clone an adult animal, it was soon agreed that these sheep would be used as recipients of embryos cloned from adult cells. Cells obtained from cultures of mammary tissue were chosen as donors. These cells were derived by Angelika Schnieke for studies of gene function in the mammary gland and were known to be stable and possess a normal karyotype. This sequence of events explains why we chose mammary cells for this experiment.

During the course of the season, lambs were derived from day-9 embryo cells, day-26 fetal cells, and mammary tissue of a 6-year-old ewe in the final trimester of pregnancy (27). At the time of their birth, the fact that the lambs were all of different genotype to the oocyte donor strongly suggested that they were clones, and this was confirmed by very detailed DNA fingerprinting (28). Although the birth weight of all of the lambs was within the range for their breed, there was a suggestion that they were unusually large, a change that had been observed in other nuclear transfer experiments (29) and to which I return below.

The success rate of nuclear transfer was and still remains rather low. The inefficiency reflects losses at all stages from early cleavage to birth and beyond. In this study, we detected 21 fetuses by ultrasound scanning at days 50–60 of gestation, but only 38% of these survived to term. A postmortem study of four of these lost fetuses was possible when dead lambs were detected by ultrasound at approximately day 110 of gestation. Two were found to have abnormal livers, but no other abnormalities were seen. There was no evidence of infection. The loss of 62% of the conceptuses is far greater than would be seen in normal husbandry (30).

The announcement of the birth of Dolly, the adult clone, attracted a great deal of interest from both the general and scientific media. Working together with the Roslin Institute and PPL Therapeutics, we had prepared thoroughly for the announcement. Knowing that *Nature* decides on Thursday which papers are to be published the following week, on the Friday before publication we sent out briefing notes describing exactly what we had done, as well as the scientific and social issues as we understood them. In addition, we prepared for a press conference on the day when the embargo ran out. Unfortunately, the story was broken prematurely by a British Sunday paper. The Deputy Director, Dr. Harry Griffin, who was responsible for public outreach, and I spent the Sunday at the Institute taking telephone calls from the United Kingdom, the East Coast, and then the West Coast of the United States. Finally, we went home before Australasia woke up. The following morning, there was a staff meeting so that we could inform the staff of what had been achieved and discuss the implications of the work with them. There were several satellite trucks in

the car park and we gave interviews to *Good Morning America* and other news programs. By that time the daily papers were full of the story. The *New York Times* sent three journalists and a photographer to report on the story. Later, we held a press conference in Edinburgh.

A majority of the media interest concerned the possibility of using our technique to clone a person. In this regard, the views of the group are well known and published in several places (31). At that time, and still today, any attempt to produce a child by nuclear transfer would seem to me to be cruel because of the significant risk of abnormal development of the conceptus leading to late-pregnancy loss, death of the newborn child, or worst of all the birth of a child with an untreatable abnormality. In one of our experiments, a lamb was born that was physically strong and active. It followed its assigned carer everywhere. However, it hyperventilated (panted) all the time. Neither the veterinary surgeons nor the pediatricians we consulted were able to offer any treatment, and after a few days, we decided that it was kinder to end the animal's life and carry out a detailed histopathology. The abnormality causing the hyperventilation was hypertrophy of the smooth muscle around the arterioles in the lungs. This experience was distressing enough in the case of an animal; imagine the feelings if the clone had been a child.

Should it ever be possible to produce healthy offspring of nonhuman primates by somatic cell nuclear transfer in a repeatable manner, there is another concern, which we believe justifies making the procedure illegal. We believe that each child should be wanted as an individual, and for all of the hypothetical justifications for production of a cloned child, there would inevitably be a disturbance to the relationship between the biological parents and the child.

By contrast, we were strong advocates of a proposal to produce ES cells from cloned human embryos because this would provide ES cells of selected genotype, either for treatment of the donor or for research. This proposal was as controversial as that to produce a cloned child. The hope was to use cells produced in this way to study inherited cases of degenerative diseases, such as amyotrophic lateral sclerosis (ALS), otherwise known as motor neuron disease. Encouragement for this approach was provided by an unexpected demonstration that ES cells derived from cloned mouse embryos closely resemble ES cells derived from embryos produced by mating (32). Progress toward that objective was slowed by the report that there is something different about primate oocytes and embryos. Removal of the second metaphase according to protocols used in other mammals disturbed development so that, following the introduction of a somatic nucleus, development ceased at the 8-cell stage when the embryos failed to initiate transcription (33). However, as this manuscript is being completed, a report has been published of modifications to the protocol that lead to blastocyst formation by cloned human embryos and ES cell derivation from them (34).

REPROGRAMMING OF CELLS

With the benefit of hindsight, we can now see that the greatest inheritance of the Dolly experiment was to make people think differently. After the experiments of Hal Weintraub and others, the consensus had been that there is only a limited opportunity to change cells from one phenotype to another. In a landmark study, Weintraub and colleagues (35) introduced into a variety of cells a transcription factor that had recently been shown to have a pivotal role in muscle formation. A cDNA coding the single transcription factor was introduced into representatives of all three germ layers: fibroblasts from mesoderm, keratinocytes from ectoderm, and hepatocytes from endoderm. This one cDNA was able to convert fibroblasts to myoblasts but was unable to convert keratinocytes or hepatocytes to myoblasts, although a low level of muscle gene expression was induced. These and other similar observations were interpreted to indicate that it would be possible to direct cell fate within germ layers but not between them.

To biologists, the great excitement caused by the birth of Dolly was the indication that, in some circumstances, it is possible to redirect fate between germ layers, indeed to pluripotency. We were one of a number of groups who set out to mimic the effect of the oocyte, but we had limited success. We found that extracts of mouse ES cells introduced after permeabilization of the cell membrane by streptolysin-O were able to induce expression of key pluripotency genes in human fibroblasts over an 8-h period. This change was associated with loss of repressive histone modifications at gene promoter regions (36).

Two groups working independently successfully devised methods for the introduction of groups of transcription factors that could induce pluripotency in mouse and human somatic cells (37–39). This likely will be one of the most important developments in biomedical research this century. It is providing exciting new opportunities in research already and will offer new approaches to cell therapy in the near future. I return to this subject later.

GENETIC MODIFICATION OF LIVESTOCK

We took the opportunity when giving interviews to advocate the use of our technique to introduce precise genetic modifications into animals. There are several reasons why this might be done. It might be to modify animals for agriculture to direct the production of biomedical proteins, or to produce genetically modified organs that could be transplanted into human patients. At Roslin, we made two more contributions to this research area.

In a further collaboration between the Institute and PPL Therapeutics, Angelika Schnieke and her colleagues used our protocol to transfer a gene to direct production of clotting factor IX into sheep (40). In this initial study, two genes were co-transfected into cells derived from a late sheep blastocyst that had previously been shown to be able to direct development to term after nuclear transfer. One gene contained the regulatory elements of the gene for the major whey protein of sheep's milk, β-lactoglobulin (3BLG), to direct expression in mammary gland of a cDNA encoding human factor IX (F9). The second gene conferred resistance to neomycin to provide an opportunity to select cells in which the transgene was present. In total, five lambs were produced that carried the factor IX transgene. Although the co-transfection did not always lead to both genes being present, this study demonstrated the feasibility of using this approach to make the process of adding a gene more efficient. By using nuclear transfer to introduce a transgene and then selecting appropriate cells, the cost of maintaining pregnant ewes carrying lambs that lack the transgene is minimized. It is also possible to use cells of the particular sex that is most advantageous for the particular project.

The possibility of being able to transplant organs from animals into people to meet the growing need for organs was attracting a great deal of interest at the time of our cloning research (41). One approach under consideration was to turn off or delete α1,3-galactosyltransferase, a gene that adds a sugar moiety to the surface of pig cells but is inactive in human cells. The galactose residue on the surface of pig cells ensures that if tissue from a regular pig is transferred into a human patient, the cells are recognized by circulating antibodies directed at that antigen, and it is destroyed within minutes by hyperacute rejection. We all have that antibody, because bacteria have this sugar on their cell surface and we form antibodies against it. Clearly, it was attractive to be able to remove the galactosyltransferase gene so that pig cells would not carry that sugar. With this in mind, we began to develop methods for nuclear transfer in the pig.

There are two technical challenges to nuclear transfer in the pig that are not encountered in ruminants. First, there is not a system for controlling the time of ovulation in pigs that is comparable to the protocols that are effective in sheep and cattle; in addition, the period of estrus is longer and harder to detect. Second, pregnancy is established only if there are more than four embryos present (42). When considering embryos derived by nuclear transfer, the majority of

embryos judged to be developing normally at the time of transfer will fail to reach term. Two innovations were made to address these concerns. Time of ovulation was determined accurately by transcutaneous ultrasonography (43). Further, we experimented with the use of hormonal treatment and the transfer of parthenote embryos to maintain pregnancy (44). Unfortunately, neither these nor other strategies were brought to fruition before Geron, who were funding this research, decided that they no longer wished to fund research to develop nuclear transfer.

MOLECULAR MECHANISMS THAT LIMIT THE EFFICIENCY OF SOMATIC CELL NUCLEAR TRANSFER

Although the proportion of cloned embryos that develop is still disappointingly low, in reality it is still a surprise that it is ever successful. The molecules that are present in the recipient oocyte are expected to modify chromatin structure and gene expression in a differentiated somatic cell nucleus to that of a newly fertilized egg able to support normal development to term. Two avenues of research led by Lorraine Young provided information on mechanisms that might lead to abnormal development: developmentally regulated changes in DNA methylation and physiological changes in fetal development after perturbations that lead to the birth of large offspring.

Other researchers had established changes in DNA methylation in the pronuclei and early cleavage stages of mouse embryos (45) and, as is often the case, had assumed that the mechanisms in all other mammals are identical to those in the mouse. In fact, a comparison of mouse and sheep embryos revealed that in contrast to mice, in sheep, no genome-wide demethylation of the paternal genome occurs within the first postfertilization cell cycle. Transfer of sperm into sheep and mouse oocytes demonstrated that sheep sperm DNA can be demethylated in mouse oocytes but, surprisingly, that mouse sperm were demethylated only to a limited extent in sheep oocytes. These results suggest an important role of the oocyte and, in addition, that the process is influenced either by a sperm-derived factor or by male pronuclear chromatin composition (46). An analysis of changes in DNA methylation in sheep embryos produced by nuclear transfer revealed a limited quantitative change in DNA methylation (47) and considerable variation in the extent of DNA demethylation during the first cell cycles. The proportion of embryos at a particular stage of development that were expected to develop to term apparently correlated with the proportion of embryos in which sufficient demethylation had occurred to establish an apparently normal level at that particular time. This led the authors to suggest that failure to achieve effective rapid demethylation was a major factor limiting development of those cloned embryos that ceased development within a few cleavages (47).

A very large and systematic comparison of calves derived by artificial insemination, embryo transfer, or nuclear transfer carried out by Granada revealed that some cloned calves are unusually large at birth (29) and prompted research to understand the biological mechanisms. A similar change had been reported following the culture of sheep embryos for just five days in the presence of serum. We chose embryo culture as our experimental system because the effects of culture are more reproducible and both simpler and easier to apply than those of nuclear transfer. A collaborative project between the Roslin Institute and a group in Aberdeen, led by John Robinson, Peter Broadbent, and Kevin Sinclair, examined the physiological effect of culture and the molecular mechanism causing the effect. These studies showed dramatic enlargement of liver, heart, and muscle in fetuses recovered at day 125 of pregnancy (48). There was no difference in expression of *IGF2* in tissues from these sheep, but expression of *IGF2R* was reduced significantly (49). Because *IGF2R* acts as a sink for the growth factor, this could account for the increased growth. Examination of the differentially methylated region in intron 2 of the gene revealed a significant reduction of methylation in the large lambs. This is expected to result in lower expression of the

gene. These two strands of research contributed important understanding of the failings of the present systems for nuclear transfer in mammals.

PRECISE GENETIC MODIFICATION OF GENES IN LIVESTOCK

Since that time, many of the objectives that we considered initially have been achieved by others. Jim Robl and his colleagues at Hematech, Inc., have modified cows so that the antibodies they produce are encoded by human genes. This was a formidable technical achievement because it involved creating microchromosomes to carry the large sequences that encode the immunoglobulins and ensure replication of the chromosome. This microchromosome was successfully introduced into cells and transmitted through many cell divisions (50). Because it is very difficult to separate human and bovine antibodies, they inactivated the bovine genes and, in addition, deleted the *PRNP* gene from the cattle to ensure that prions could not possibly be present in any clinical product (51). In the past, the mouse was the only species in which it was possible to introduce precise genetic changes. Specific mutations have been introduced into mice with the aim of creating models of human genetic diseases, and experience has shown that differences between mouse and human limit the value of these models. Researchers can now choose the most appropriate among several different species. For instance, they have established effective models of cystic fibrosis and Alzheimer's disease in the pig (52, 53). With the aim of transplanting pig organs into human patients, several genetic modifications have been made, including the deletion of galactosyltransferase. This significantly increased the period for which the heart functioned after transplantation into baboons to a median of 78 days (range 2–6 months), but this is still not a clinically useful period (54).

LEADERSHIP OF THE CENTRE FOR REGENERATIVE MEDICINE

By 1995, I was approached in regard to a chair in reproductive science in the Division of Reproduction and Development in the Medical School in Edinburgh and was glad to make the move. A few months later, when Austin Smith informed the university that he was planning to move to Cambridge, I was invited to take over the leadership of the Centre for Regenerative Medicine in the University. Edinburgh had first-class stem cell biologists in the College of Science and Engineering, led by Austin in the Institute for Stem Cell Research, and several clinicians in the medical school who either worked with or wished to work with stem cells. Despite the fact that the two groups were only one or two miles apart, there was almost no interaction. There was a wish to bring these two groups together in a purpose-designed facility adjacent to the Royal Infirmary, a major, 900-bed research hospital and a large section of the medical school in the new campus at Little France. Over the following five years, with the guidance of the head of the College of Medicine and Veterinary Medicine, Professor Sir John Savill, we established a virtual center; began the process of integrating the two groups; and designed and built the facility, which we moved into in October 2011. During that period, we also gained recognition as a Medical Research Council Centre. I was immensely privileged to have the role of leading this change.

STUDIES OF AMYOTROPHIC LATERAL SCLEROSIS

It was during this period that Takahashi and Yamanaka announced their method for production of pluripotent cells by the introduction of just four transcription factors, first in mouse and then in human (37, 39). These were clearly groundbreaking studies that would make it easier to study inherited diseases. During the next few years, a group that included Chris Shaw at Kings College,

London, George Daley at Harvard, Siddharthan Chandran at Edinburgh, Tom Maniatis at Columbia, Steve Finkbeiner at San Francisco, and myself developed a collaborative project to study ALS. We elected first to study a case in which the patient had inherited a mutation in the gene *transactive response DNA binding protein* (*TARDBP*), which encodes the protein known as TDP-43. We chose this gene because unusual aggregates containing this protein are found in motor neurons of more than 90% of cases of ALS, even though mutations in *TARDBP* are present in less than 2% of cases (55). The fact that it is involved in such a wide range of circumstances suggests that it may have a causative role, in which case remedies identified in research with inherited cases of mutation in *TARDBP* might also be effective in other cases.

We derived iPS cells from a patient by using the Yamanaka factors and differentiated them to motor neurons and astrocytes. Detailed analyses were made to confirm the nature of the cells and assess gene expression (56). These cells were then cultured separately and in coculture to determine their survival in culture over a 10-day period. The cells were transfected with a reporter gene and monitored on a robotic microscopy system every 24 h for loss of the reporter or loss of normal structure. When compared with equivalent cells from a control, healthy person, the cells carrying an ALS mutation were significantly more likely to die. There was no effect of coculture on survival of either type of cell, an observation that contrasts with reports that, in cases caused by mutation in SOD1, glia act to hasten the death of motor neurons in coculture.

So far, these observations have been made with cells from only one donor, and they require confirmation. Further, to confirm that the difference arises from the mutation, it is essential that the mutation is corrected and survival compared between cells from the healthy control and the ALS case and the corrected cells from the patient. If it is confirmed that cells from patients with inherited ALS are liable to die in culture, this will provide an easy assay of the effects of small molecules on survival. Any that prolong survival would be candidate medicines for treatment of the disease. This treatment would be used to limit or ameliorate the harmful effects of the mutation, not to correct the mutation. There are literally hundreds of degenerative diseases that reflect the influence of a harmful mutation, and very few have effective treatments; fortunately, many are extremely rare. In principle, if the tissue that is harmed in the disease can be produced in tissue culture, this approach can be used to study the molecular mechanisms that cause the symptoms of the disease and so provide a means of searching for effective drugs.

FUTURE AMBITIONS

In my present role as an emeritus professor in the Centre for Regenerative Medicine, I would hope to be a collaborator in three projects that will reach a successful conclusion over the next few years. One would be to see the first drugs to treat ALS available to the general mass of patients. This is a very cruel disease, and it would be a joy to see the harmful effects reduced or delayed in some way. The second would be to bring forward a cell therapy. A group of us in another international partnership are beginning a campaign to establish libraries of cells from donors who are homozygous at the HLA antigens. It is estimated that cells from approximately 150 carefully selected donors would provide a useful immunological match for over 90% of the UK population (57), and similar numbers are likely to be required elsewhere. It is important that the terms of donor consent, procedures for culture and storage of the donor cells, method of reprogramming, and assessment of the iPS cells are agreed upon to allow free movement of cells around the world. This library would be an invaluable resource for those who are developing the use of iPS cells in cell therapy. Finally, I would very much like to be involved in a project to produce hepatocytes that could be used to provide temporary support for those patients who cannot receive a liver by transplantation. They might be introduced into the peritoneal cavity temporarily or be used outside the

body in a method comparable to dialysis for kidney failure. Suitable cells can now be produced in the laboratory, but there is a need to expand cell numbers in clinical-grade protocols and to demonstrate in preclinical trials that the cells can offer the needed benefit. This approach takes advantage of the liver's ability to repair itself if given the opportunity.

DISCLOSURE STATEMENT

My wife and I own shares in the Geron Corporation of Menlo Park, California.

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One of the great joys of a research career is the opportunity to conduct collaborative research, which involves working with people with different experience and skills and the exchange of ideas through discussions in frequent meetings. It is in this way that new ideas are often developed. I am deeply grateful for the stimulation, instruction, and encouragement of all those collaborators and colleagues who are mentioned in this essay.

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