Nuclear Cloning and Direct Reprogramming: The Long and the Short Path to Stockholm

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Forum

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The two winners of the 2012 Nobel Prize in Physiology or Medicine share more than just this honor; they are both also fearless adventurers, in science and beyond.

This year's Nobel Prize in Physiology or Medicine was awarded to John Gurdon and Shinya Yamanaka for solving key problems of biology. Their work provided fundamental insights into how an egg gives rise to a complex organism consisting of many different cell types and has transformed our understanding of nuclear reprogramming, of embryonic development, and of cellular differentiation. These are issues that have a long history and the reader is referred to a comprehensive review by Graf (2011) on cellular plasticity and a summary of current issues of the reprogramming field by Yamanaka (2012). Here I will give a personal view on how the discoveries of Gurdon and Yamanaka have shaped our understanding of basic mechanisms of development and how stem cells will revolutionize the way we investigate human disease and establish novel treatment strategies.

For the longest time, biologists tried to unveil the process of how a fertilized egg creates an organism that consists of hundreds of specialized cell types that are all expressing different sets of genes. Development of an embryo to the adult was seen as a unidirectional process, and the differentiated state of specialized cells like skin or liver cells as fixed irreversibly. How specific combinations of proteins could be synthesized and maintained in different cell types needed an explanation. Two competing hypotheses were envisioned. August Weissmann and Wilhelm Roux proposed in the late 19th century that cells become different by selective loss of genetic material that was not needed and retain only those genes essential for the function of the respective tissue, as has been seen in some animal species. The second hypothesis posed that differentiated cells may retain all genes but regulate their expression in a tissue-specific manner. Gurdon and Yamanaka's seminal work resolved these questions. We now know that the genome is conserved during development, and that the differentiated state is reversible. Moreover, in the process Gurdon and Yamanaka shed light on the nature of stem cells and pluripotency.

John Gurdon: Is Genetic Information Lost in Development?

Though interested in science from a young age, Gurdon's entrance into biological science was highly discouraged by his biology teacher, who said of Gurdon, "While having ideas to become a scientist, this is quite ridiculous as judged from his present showing: he can't even learn simple biological facts and would have no chance of doing the work of a specialist and it would be a waste of time, both on his part and of those who have to teach him." A prophecy that now makes us smile!

A stringent prediction of the Weissmann-Roux concept was that nuclei of differentiated cells would lose the ability to generate a new organism. In contrast, if all genes were retained and the process of differentiation was reversible, a somatic nucleus would maintain the potential to form a new organism when transplanted into the egg. In 1952 Briggs and King performed the first successful nuclear transfer (NT) experiments in the frog *Rana pipiens*. When nuclei from cleavage embryos were used as donors they obtained swimming tadpoles, but later stage nuclei had lost this potential and formed only abnormal clones. They concluded that differentiation involves irreversible nuclear changes and the production of animals by nuclear cloning from somatic donors would be impossible, and that conclusion became generally accepted in the field until John Gurdon made his crucial discovery.

Working as a student in the laboratory of embryologist Michail Fischberg, Gurdon decided to test the Weissman-Roux hypothesis in *Xenopus* rather than *Rana* eggs. For practical reasons this was an excellent choice because *Xenopus*, in contrast to *Rana*, can produce eggs all year round. His early experiments challenged the conclusions of Briggs and King: nuclei derived from intestinal cells of tadpoles, when injected into the egg, were able to direct development to mature adults (Gurdon, 1960; Gurdon and Uehlinger, 1966). This seminal discovery made a persuasive case that development does not in fact involve irreversible genetic changes. It took 30 years for Keith Campbell and Ian Wilmut to repeat this experiment in mammals by generating Dolly, the first cloned mammal derived by transfer of an adult somatic nucleus into an enucleated sheep egg (Wilmut et al., 1997). To prove that nuclei of mature adult cells rather than tissue stem cells retained totipotency, Gurdon used expression of tissue-specific markers in the donor cells. However, because cloning was so inefficient the question of whether nuclei of mature adult cells were indeed totipotent was only settled later when it was shown that all cells of cloned mice harbored the appropriate IgG or T cell receptor gene rearrangements when derived from mature B or T donor cells (Hochedlinger and Jaenisch, 2002).

The molecular mechanism of nuclear reprogramming by the egg has been of great interest to biologists and has largely remained unsolved even today. Because of the egg's small size, biochemical analyses of the reprogramming process in the mammalian egg are difficult.

Gurdon realized early that the *Xenopus* oocyte, in contrast to the mammalian system, would provide a better opportunity. He used the *Xenopus* egg as a living test tube and showed, for example, that nuclei from differentiated cells, when transferred into the oocyte, silenced genes expressed in the somatic donor cell and activated oocyte-specific genes (De Robertis and Gurdon, 1977). The important conclusion was entirely consistent with differential gene expression driving development because it showed that genes, which had become inactive during cell differentiation, can be reactivated by normal components of the oocyte cytoplasm in the absence of cell division. These experiments were performed in the 1970s at a time before today's routine molecular tests such as Southern analysis, PCR, or genomic analyses had been invented, yet the conclusions have stood the test of time. In more recent years Gurdon introduced mammalian nuclei into the *Xenopus* oocyte and, using modern experimental tools, has embarked on defining molecular events of early stages in nuclear reprogramming. Given the different developmental strategies of frog and mammal it will be important though challenging to confirm that conclusions from these interspecies experiments accurately reflect what happens to the mammalian nucleus when transferred into the mammalian egg.

When I first read about NT, I was deeply impressed by the elegance and the boldness of Gurdon's experiments and later by the clarity of his lectures. It may come as no surprise that he is as passionate and fearless outside of science. He used to bicycle 20 km to work, still plays tennis and competitive squash, and has made adventurous treks to the high mountains. For example, in search of Meconopsis, a beautiful poppy plant that only blooms in the monsoon season at an altitude of 14,000 feet, he embarked on long and treacherous treks into the high mountains of India. In the 1990s he trekked into the mountains of New Guinea, completed the Haute Route (a demanding 7 day ski trek though the high French and Swiss Alps), and climbed Mt. Harvard, one of the highest peaks in the Rockies (see photos). Ten years ago he decided it was time to learn parachute jumping, an idea provoked by one of his students who had become an expert in this activity. His first jump was 1 day before his daughter's wedding and 1 week after a lethal accident had occurred due to the failure of a chute to open, all circumstances that, as he told me, caused his wife some concern. A water skiing accident necessitating hip replacement has not dampened his resolve to plan a serious hiking trip into the Himalayas to see rhododendrons in bloom. Today, at 79 years of age, Gurdon retains the skills to inject nuclei and comes every day to the lab to do his experiments. He occupies a small office that also houses his microscope. Because the University does not allow the placement of scientific instruments in an office, he has declared his office to be his laboratory, which just happens to also contain his desk.

John Gurdon as a mountaineer in the 1990s during the Haute Route (a strenuous 7 day ski trek crossing high mountains from Chamonix in France to Zermatt in Switzerland) (top), and on Mt. Harvard, which is among the highest peaks in the "collegiate" range in central Colorado (bottom). Photos courtesy of J. Gurdon.

Gurdon's classic NT experiments established for the first time that nuclei of differentiated cells remain totipotent and retain the potential to produce a new organism. His experiments did not address whether differentiated cells can be reprogrammed back to an undifferentiated pluripotent state. The answer to this question would come from the work of Shinya Yamanaka.

Shinya Yamanaka: How Does the Egg Reset the Genome?

In the late 1990s, two breakthroughs stirred the scientific community as well as the public: the generation of Dolly, the first mammal cloned from an adult donor cell, and the derivation of human embryonic stem cells (ESCs) by Jamie Thomson (Thomson et al., 1998). Together these discoveries raised the possibility of customized therapy for human degenerative ailments such as Parkinson's or heart disease with what was dubbed "therapeutic cloning." The idea was that patient-specific pluripotent ESCs could be obtained by NT of a skin cell nucleus into a human egg, which then would be differentiated to the cell type that was defective in the patient. The differentiated cells would be transplanted back into the patient to cure the underlying disease without the complication of immune rejection. While therapeutic cloning was shown to work in animals, its application to humans faced serious technical, practical, and ethical obstacles that subsequent work has still not been able to overcome (Hyun, 2011). Importantly, it was inconceivable that sufficient numbers of human eggs could ever be obtained for

Shinya Yamanaka as a student with lab members at Osaka City University Graduate School in 1992 (left) and as rugby player while a medical student at Kobe University in 1985 (right). Photos courtesy of S. Yamanaka.

any medical application. Finally, serious moral objections were raised to using human eggs and cloned embryos for research and therapy. Thus, the only option was to understand how the egg accomplishes the resetting of the somatic genome to a pluripotent state, which would circumvent the need to use human eggs in the process. The mechanism of nuclear reprogramming during cloning became a hot research aim that captivated many laboratories. It is here where Yamanaka made his groundbreaking discovery.

During his high school years, Yamanaka practiced judo and earned a black belt. He enrolled in medical school, where his focus was on playing rugby (see photos) at the expense, as he told me, of attending classes. Serious practice of judo and rugby is tough on the body, and he suffered numerous bone fractures necessitating intimate and painful exposure to medicine as patient. These experiences convinced him to pursue a medical career and become an orthopedic surgeon. However, medical school was not to his liking: he had to follow textbooks and supervisors closely. Nevertheless, he finished medical school and got his M.D. degree, only to realize that surgery and the manual dexterity involved were not his calling. Instead, he decided to study for a Ph.D. in pharmacology. Excited by the isolation of human ESCs and their potential for medicine, he set out to define the molecular determinants of the pluripotent state.

The mammalian egg is about 1,000× larger than a somatic cell and harbors numerous gene products that are crucial for the early development of the embryo and, it was assumed, for reprogramming the somatic nucleus after NT. It was a daunting task to identify which of the many proteins present in the egg might be responsible for resetting the somatic cell genome back into an embryonic state. In the early 2000s several laboratories had defined the key factors responsible for the maintenance of ESCs with the transcription factors Oct4, Sox2, and Nanog being at the top of an autoregulatory gene expression circuitry required for pluripotency and self-renewal. While this discovery suggested to many that expression of these genes in somatic cells would be essential to induce reprogramming, they surely were not deemed sufficient on their own—the process had to be far more complex.

Yamanaka dropped a bombshell on the field at the 2006 ISSCR meeting in Toronto when he announced that only four transcription factors were needed to reprogram a somatic nucleus to pluripotency. Kazutoshi Takahashi, a student in his lab, had performed an experiment that seemed unlikely to succeed. From a list of genes expressed in ESCs, but not in somatic cells, they had selected 24 candidates, which were packaged into retroviral vectors and cotransduced into fibroblasts that carried a neo resistance marker in the Fbx15 gene. Fbx15 is downstream of Oct4 and is not expressed in fibroblasts but is active in pluripotent ESCs. If any combination of the 24 candidate genes could induce reprogramming, the rare reprogrammed cell would become neomycin resistant and thus could be isolated (Takahashi and Yamanaka, 2006). From the point of view of a retrovirologist, as I am by training, this scheme seemed bold and unlikely to work because cotransduction of multiple genes into a single cell would be exceedingly unlikely. However, because of the strong selection, they were able to isolate reprogrammed cells designated as "induced Pluripotent Stem" cells (iPSCs) by transduction of only four genes: Oct4 and Sox2 and the oncogenes c-Myc and Klf4. While the importance of Oct4 and Sox2 was more or less predictable, the choice of c-Myc and Klf4 was, in my judgment, brilliant. As it turned out later, the two oncogenes are not required for reprogramming, but they made the process more efficient, and made detection of rare reprogrammed cells possible.

Yamanaka's talk at the ISSCR meeting was met with disbelief and skepticism by most: impossible that reprogramming was that simple! Knowing Yamanaka and his rigorous scientific standards, I was convinced immediately. But the iPSCs reported in the initial landmark publication were not equivalent to ESCs: they had not activated the endogenous pluripotency circuitry and were unable to generate chimeras. A year later, using a modified approach, three groups, including Yamanaka's, reported the generation of iPSCs that were indistinguishable from ESCs. Confirmation from three independent labs convinced the community and led to an explosion of the reprogramming field. Five months after that, several groups reported reprogramming of human cells, attesting to the robustness of the Yamanaka approach. The prospect of using human iPSCs for disease research and for cell-based therapy has electrified the scientific community and beyond, generating much excitement and expectation among the general public.

Nuclear Cloning, Direct Reprogramming, and Transdifferentiation

Nuclear reprogramming by NT versus reprogramming initiated by transcription factors raised an interesting question: how similar or different are the molecular mechanisms that reset the genome? Has direct reprogramming by transcription

factors *really* taught us how the egg achieves epigenetic resetting of the somatic nucleus after NT?

It had been established early on that pluripotency factors are reactivated within one or two cell divisions after NT into the mouse egg or without DNA replication after transfer of somatic nuclei into the *Xenopus* oocyte (see, for example, De Robertis and Gurdon, 1977, and later work from the Gurdon lab). In contrast, direct reprogramming is inefficient and iPSCs appear only after multiple cell divisions. Thus, it was postulated that the molecular mechanism in NT and direct reprogramming would be very different: reprogramming by NT might be a ''deterministic'' process resulting in rapid hierarchical activation of pluripotency genes, in contrast to iPSC formation that requires multiple ''stochastic'' or probabilistic epigenetic events that only accumulate after many cycles of DNA replication. However, there is still much to learn about the mechanisms that underlie reprogramming, and recent studies have suggested that direct reprogramming takes place in distinct stages and may well have a more deterministic component than previously assumed.

The concept that ectopic expression of transcription factors can reprogram somatic cells to pluripotency was soon extended and applied to converting cells from one somatic lineage to another, a process called ''transdifferentiation.'' The idea that key transcription factors can change the differentiation state of a cell had been established in principle some time before (as described by Graf, 2011). For example, conversion of fibroblasts into muscle cells by transfection of MyoD by Harold Weintraub or Pu-1-mediated conversion of lymphoid cells into macrophages by Thomas Graf were among the first demonstrations that a single transcription factor could convert one cell type into another. Although the early experiments achieved conversion of cells within a given lineage, more recent studies have applied the Yamanaka approach to transdifferentiate cells of one germ layer to that of another, such as fibroblasts to neurons or liver cells. The approach has even been extended to the in vivo conversion of exocrine to endocrine pancreas cells (for details of these and the early experiments, see Graf, 2011). Successful transdifferentiation was inspired by Yamanaka's discovery of inducing reprogramming with several transcription factors and shows that the iPSC approach has significance beyond just the conversion of somatic to pluripotent cells.

What Lies Ahead?

The most immediate application of iPSC technology is the study of human disease: iPSCs derived from a given patient carry all genetic determinants that have contributed to the disease and thus represent an unparalleled system to study the etiology of diseases in the Petri dish. They also offer screening for potential drugs and might be used for cell-based therapy at some point in the future. Although technical issues pertaining to the significance of genetic and epigenetic differences between ESCs and iPSCs and a number of safety concerns still need to be resolved, and robust protocols for the differentiation of iPSCs into functional cells need to be developed (for detailed discussion see Yamanaka, 2012), it is a safe prediction that this approach will change the way we will investigate and treat diseases.

The direct reprogramming of somatic cells with only a few transcription factors has fundamental medical implications and arguably represents the most important finding in the stem cell field since Gurdon's demonstration of reprogramming by nuclear transplantation. It took Gurdon 50 years after his original discovery and Yamanaka a mere 6 years to be recognized by the highest scientific honor. This difference, I believe, reflects the breathtaking progress in the stem cell field following the first report on iPSCs, and the likely unprecedented impact that iPSC technology will have on medicine in the years to come.

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