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New Developments in Stem Cell Biotechnology

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The subject of stem cells has attracted a great deal of interest in the public during the last twelve years. Indeed it brings about the hope of a novel medicine through which cells in the adult organism that are deficient or subjected to massive death could be replaced by healthy ones. With the increase in longevity in industrialized countries, such instances, resulting from degenerative diseases, are more and more common. This *regenerative medicine* would complement therapeutics relying on surgery, chemistry and antibodies, which are one of the most important legacies of the 20th century.

During the last four decades it has been recognized that stem cells are present in virtually all tissues in adult vertebrates and are a source of youth, since their role is to replace cells which regularly die during the lifetime of the individual. Moreover, vertebrate embryos are entirely made up of stem cells at the early stages of their development. This pluripotent state of embryonic cells is transitory, but can be captured thanks to the spectacular advances in the biotechnologies during the last decades. It is now possible to maintain this particular *stemness* state in culture, thus generating permanent cell lines, endowed with the properties of this pivotal and intriguing type of cells.

The term *stem cell* can be found in the scientific literature of the first half of the 20th century. However, its definition was not clear until it was based on rigorous experimental criteria. This was achieved in the 1960s thanks to a series of studies that demonstrated the mechanisms through which the replacement of blood cells, whose normal lifespan is short, takes place.

In this article I will review the seminal work that has led to the scientific definition of a stem cell and then go through the successive breakthroughs that have stood out as landmarks in the field over the years and have led to the state of the art of today.

The definition of stem cells

As a general rule, the cells of the body that differentiate to fulfil definite functions have a lifespan shorter than that of the organism and are, therefore, subjected to constant and periodic renewal. Cell turnover varies considerably from one type of tissue to the other. It is rapid for the epithelium lining the

intestinal lumen whose cells are replaced every three to five days, or for the skin epidermis that, in humans, is renewed every twenty-one to thirty days. In the blood, the erythrocytes survive one hundred and twenty days after they have reached their fully functional state.

In the nervous tissues in contrast, most neurons are not renewed during lifetime in Mammals except in some areas such as the olfactory bulb in rodents and regions of the brain associated to memory (e.g. the hippocampus).

The concept of stem cells and the demonstration of their properties emerged from the observation that victims of the Hiroshima and Nagasaki nuclear bombs, who did not die at the time of explosion, died ten to fifteen days later in a state of advanced anaemia, with severe depletion of the bone marrow and spleen. The bone marrow had previously been recognized to be a site of production, proliferation and maturation of blood cell progenitors, and this effect was attributed to the sensitivity of dividing cells to ionizing radiations.

Experiments were conducted in the mouse that reproduced this effect of irradiations on the blood cell lineage. It was shown that irradiated mice could be rescued if they received bone marrow cells from histocompatible donors. Rescue was complete provided that donor cells became stably engrafted within the recipient spleen and bone marrow, thus providing a long-term reconstitution of the irradiated recipient hematopoietic system by the injected cells.

One of the consequences of this treatment was the fact that the spleen size, which shrank after the irradiation, regained its normal volume after the hematopoietic reconstitution.

At that time, two views were held concerning the cells that were at the origin of the renewal of the blood cell lineages. One proposed that each type of blood cells (e.g., erythrocytes, and the different sorts of leucocytes) were produced by a distinct undifferentiated progenitor. This was held by the tenants of the *polyphyletic origin* of the blood cells. According to the other view (*monophyletic*), one single pluripotent progenitor was at the origin of the various types of blood cells. The problem was solved by experiments carried out in the early 1960s by two Canadian haematologists working in Toronto, James E. Till and Ernest A. McCulloch [1]. Their experimental design consisted in reducing as much as possible the number of bone marrow cells able to reconstitute the blood cell system of the irradiated recipient. This goal was attained with 10^5 bone marrow cells. This experimental protocol led to the formation on the recipient's shrunken spleen of individually distinguishable bumps instead of the general swelling of the organ observed after the injection of larger numbers of cells.

They could show that each of these bumps, which contained all kinds of blood cells (except lymphocytes), corresponded to the engraftment of one single progenitor of donor origin. They were subsequently able to demonstrate

that the progenitor cell at the origin of the colony had also produced in its progeny undifferentiated cells similar to itself, which were able to produce new colonies if subjected, *in vivo* or *in vitro,* to appropriate conditions.

These experimental data led to the denomination of these blood cell lineage progenitors as *Hemopoietic Stem Cells* (HSC). These HSC were endowed with the following characteristics:

HSC are undifferentiated, divide asymmetrically and give rise to a cell similar to themselves (which remains undifferentiated and slow dividing) and to another cell with high proliferative potential, which can yield various phenotypes of differentiated cells.

In other words, stem cells are undifferentiated, pluripotent and able to self-renew, thus forming a reserve of cells able to maintain *homeostasis* in adult tissues by renewing cells that disappear through normal cell death.

One can consider the characterization of the hematopoietic stem cells as the *first breakthrough* discovery in the history of the stem cell field.

Apoptosis or normal cell death

One of the major advances in the field of cell biology in the second half of the last century was the discovery of the genetic mechanisms leading to natural cell death, also designated as *Apoptosis*.

Studies carried out on a Nematode, *Caenorhabditis elegans,* revealed that all living cells possess a gene network that enables them to commit suicide. Thus, these *suicide genes* need to be inhibited for the cell to be able to survive. Environmental signals such as growth and survival factors counteract the intrinsic cellular apoptotic machinery.

Cell death by apoptosis is unobtrusive, it starts by fragmentation of its DNA and then of its cytoplasm, and the cellular debris of the dying cells is rapidly absorbed by the neighboring cells. This is the reason why apoptosis had not been described before.

This process plays a major role during development, which involves the production of cells in excess. It is one of the means through which shaping of the organs and of the body is achieved. Moreover, it is a natural barrier against the development of tumors since, when a cell becomes abnormal by mutations paving the way to cancer, its cell death program is most often activated. This role is further attested by the fact that mutagenesis targeted to genes involved in apoptosis in the mouse markedly increases the incidence of tumors.

Cell death by apoptosis is involved in *tissue homeostasis*, which is the equilibrium between elimination of aged or abnormal cells and their replacement by new cells. The latter role belongs to the stem cells present in virtually all adult tissues.

The origin of the adult stem cells

Experiments carried out on the mouse in the 1960s have demonstrated that, at its early stages of development (i.e. morula and blastocyst stages), the mammalian embryo is composed of a clump of cells that have stem cell characteristics: they are pluripotent and able to self-renew [2]. At the blastocyst stage, the germ is composed of an epithelium that becomes the placenta (after implantation of the conceptus in the uterus) and lines a cavity in which sits an inner cell mass (*ICM*) from which the embryo develops. The cells of the *ICM* are all equivalent and each of them is able to produce all the differentiated cell types present in the adult body. Thus, one single cell of the *ICM* of an 'A' strain of mouse (with black fur), introduced within the blastocyst of a 'B' (white colored) strain recipient at the same stage, yields a chimeric mouse all tissues of which are composed of a mosaic of A and B cells. This is evident from its fur, which exhibits black and white hairs.

This early stage, where all embryonic cells are pluripotent and equivalent, is *transitory* and ends with the process of *gastrulation*, which leads to the formation of the three germ layers: *ectoderm, mesoderm, endoderm.* In each of these layers the potentialities of the embryonic cells become restricted to a defined set of phenotypes that will characterize the organ and tissue that they respectively yield. In each of these organs and tissues a reserve of stem cells subsists. These will remain undifferentiated and, later on, ensure the renewal of the differentiated cells that have reached the end of their normal life span.

These stem cells will, in the adult, subsist as discrete populations located within a 'niche' in which they will be 'protected' and maintained in an undifferentiated, pluripotent state by environmental factors. These adult stem cells are very few and, to a certain extent, specified since they produce only cells of the same type as those of the tissues they belong to.

Adult stem cells have been found in virtually all types of tissues, even in the brain and spinal cord where no new neurons were supposed to be produced after birth in mammals and birds. In fact, a certain level of cell renewal exists also in the nervous tissue and neural stem cells have been characterized in both the central (brain, spinal cord) and the peripheral nervous systems (CNS, PNS).[1]

One can consider that, in the history of the stem cell subject, the discovery of the HSC is the conceptual acquisition upon which sits the whole field

[1] For more information see *Des chimères, des clones et des gènes*. (2000) Odile Jacob Ed. and *Cellules souches, porteuses d'immortalité*. (2007) Odile Jacob Ed., by Nicole Le Douarin.

that developed later on. Twenty years later a second step took place that considerably widened its interest owing to the perspective of the potential applications it offered. This step, which pertains to the biotechnologies, consisted in 'capturing' the transitory state of pluripotency exhibited by the early mammalian embryonic cells to make it permanent. This technology has enabled to immortalize embryonic cells in a normal state in which they remain still capable of differentiating in all the cell types encountered in the adult mammalian body if provided with appropriate conditions.

The generation of Embryonic Stem Cells

In 1981 two laboratories [3] published a striking result: cells of mouse embryos of the 129 strain could be cultured permanently while remaining in the same pluripotent and undifferentiated state they exhibited in the inner cell mass. This was achieved by the particular culture conditions provided by the co-culture on certain feeder layers. If withdrawn from this environment and subjected to regular culture conditions these cells were able to differentiate in various cell types, as do cells of normal *ICM*. They were also endowed with self-renewal capacities, were pluripotent and represented the *in vitro* capture of a transitory developmental stage. For these reasons, they were designated *ES cells* (standing for Embryonic Stem cells).

For many years, *ES cell* lines could be successfully established from embryos of a particular strain of mice, the 129 strain only. Various lines of *ES cells* available were used as tools for genetic experiments in the mouse. They were namely instrumental to produce gene targeted mutations through homologous recombination, a pivotal technique to investigate the functions of genes that were currently discovered and cloned at that time by genetic engineering.

For many years the numerous attempts made to obtain *ES cell* lines from embryos of other mammals failed. But, seventeen years after mouse ES cell lines were established, James Thomson of the University of Wisconsin succeeded in deriving *ES cells* from Rhesus monkey first and from human embryos, provided to him by an *in vitro* fertilization clinic [4].

Human ES cells and the perspective of a regenerative medicine

James Thomson's experiments were reproduced by other laboratories in the world, and their results aroused a great deal of interest among the general public. The characteristics of the mouse *ES cells* were shared by human ones: one could establish permanent, virtually immortal, cell tines of human *ES cells* that remained pluripotent and could be led to differentiate *in vitro* into a

large number of cell types, including neurons, cardiomyocytes, vascular endothelial cells, striated muscle fibers, tendons, bones, cartilages… The possibility of using them for regenerative therapy in patients was then open.

Several problems however were raised by the use of human *ES cells* for this purpose.

Some are biological while others are ethical in nature.

The former concern the fact that if differentiated cells obtained from human *ES lines* are introduced into a patient, they will be subjected to immune rejection from the recipient. Ideally, the grafted cells should be 'customized' for each patient and therapeutic cloning was proposed as a method to circumvent this difficulty. Therapeutic cloning involves the substitution of the nucleus of a human oocyte by the nucleus of one of the patient's somatic cells. This technique, also designated as 'nuclear transfer', turned out to be of extremely low efficiency in mammals (mouse, sheep, cow etc.) on which it has been practiced and was unsuccessful in the few cases in which it has been applied to a human oocyte.

Moreover, it raised ethical problems of two kinds: one is the fact that it necessitates a large amount of human oocytes taken from young women, a highly unethical practice. The second is that it was argued that the improvement of the cloning technique could lead to reproductive cloning, which is generally considered as unacceptable.

Another problem, biological in nature, resides in the fact that the cultures of differentiated cells derived from *ES cells* might be 'contaminated' by pluripotent stem cells at the time they are introduced into the patient. These cells are prone to develop tumors when subjected to an adult environment.

Finally, the derivation of ES cells from a human embryo is considered by certain people as unethical, since it interrupts the development of a human being. Such is the position of the Catholic Church for whom the human nature of the conceptus starts from the moment when the two gametes fuse and form a zygote. Such a position does not hold for other religions, such as the Jewish, for which 'humanity' is acquired by the embryo only when it has reached a certain stage of development, about 40 days after fertilization.

Researchers have proposed several possibilities to circumvent these problems. One of those, for example, was to remove one single cell from an 8-cell stage human embryo and, through a biotechnological 'tour de force', derive an *ES cell* line from it. The remaining 7-cell-embryo is able to safely pursue its development as shown in routine techniques used for antenatal diagnosis.

The most spectacular result in this area was the recent demonstration that adult differentiated cells can be reprogrammed and reacquire the characteristics and potentialities of embryonic cells.

Rejuvenating adult differentiated cells

The increasing interest devoted to stem cells has led researchers to investigate the genetic characteristics of the 'stemness' state. What are the genes activated in these cells and responsible for their unique properties: undifferentiated state, pluripotency and self-renewal capacities? Several laboratories have attacked this problem using diverse types of stem cell lines and, although some differences arose in the lists of genes, the results converged on about 20 that turned out to be activated in virtually all the stem cell lines studied.

The laboratory of Shinya Yamanaka, then at the Riken Institute in Osaka, produced its own list of 24 genes and transfected cultured mouse skin fibroblasts with these genes through retroviral vectors.

They used a selection system based on the insertion of a resistance to the neomycin gene under the control of the promoter of a gene expressed in *ES cells* but not in fibroblasts, in order to recognize the cells that had been reprogrammed by the factors. The remaining cells of the fibroblast type died. Rare events of reprogramming of the fibroblasts nuclei occurred which led to the growth of colonies with the morphology of *ES cells*.

Shinya Yamanaka, with his co-worker Kazutoshi Takahashi, could obtain the same reprogramming of the fibroblasts into *ES-like* cells by transducing only four of these genes which turned out to be necessary and sufficient to produce this effect: *oct4, Klf4, c-Myc* and *Sox2*.

These genes are all transcription factors, regulating the activity of other genes.

A first report on these results appeared in 2006 and was followed one year later by an article reporting that the same reprogramming could be obtained with human fibroblasts [5].

The stable cell lines resulting from these experiments were designated as *iPS cells* for induced Pluripotent Stem cells. The *iPS cells* were found to express all the 24 genes including *Nanog*, which is often used as a 'marker' for the ES cell state. The gene expression profile of the *iPS cells* was found to be very similar to that of ES cells – although not identical – but very different from that of the original fibroblasts.

All the tests which are known to characterize *ES cells* and cells of the *ICM* were positive in *iPS cells*: formation of teratomas in adults; *iPS cells* can be led to differentiate into all tissues type cells, they form viable germ line chimeras when introduced into blastocysts, can support the complete development of an organism as shown by their capacity to yield viable mice entirely constituted of *iPS derived cells* in the tetraploid complementation assay. In this assay, *iPS cells* are introduced into the blastocyst of a mouse embryo whose cells are tetraploid. The placenta of these mice survives up

to term but the embryonic cells die progressively during the course of development; only the diploid cells that have been introduced into the blastocyst survive, thus giving rise to a normal mouse.

Since 2007 many laboratories in the world have switched to this new research line and an impressive number of results have been obtained.

Reprogramming of a large variety of differentiated cells has been achieved. Hemopoietic cells, including T and B lymphocytes and hematopoietic stem cells, could be an attractive type of cells for the generation of iPS for therapeutic purposes, liver cells, stomach epithelium, pancreatic β cells, and human keratinocytes. For example, Juan Carlos Izpisúa Belmonte has been able to derive lines of *iPS cells* from a single human hair. Neural progenitor cells can be induced into *iPS cells* without *Sox2* that they already express.

It seems therefore that reprogramming is a universal process that can be obtained from differentiated cells belonging from the three germ layers.

iPS cells have also been derived from differentiated cells of various other species of rodents (rats) or Primates.

All these results are very encouraging as to the possibility of devising novel techniques for the onset of an efficient regenerative medicine. *iPS cells* can fulfill the requirements of 'customized' cells that will not trigger an immune response from the recipient in which they will be introduced, since they can be derived from the own cells of the patient. However, their use still raises certain problems. Experiments carried out in mice have shown that chimeric mice, which are made up of a mosaic of normal and iPS derived cells, often develop tumors. This was attributed to the retroviral vectors used for gene transduction. Such vectors become inserted randomly into the host cell DNA. They may be positioned in critical locations capable of activating endogenous oncogenes. Moreover, *c-Myc*, which is one of the four genes introduced into adult cells, is itself an oncogene, overexpressed in most spontaneous tumors. Its localization within the host DNA may result in its overactivation, thus also being a cause for tumor formation.

Several laboratories are now developing methods to reprogram adult cells which would avoid the difficulties presently encountered.

In conclusion, one can consider that, following the pioneering work of Martin Evans and Gail Martin in 1981, the work of Shinya Yamanaka and colleagues must surely be regarded as the single major advance in the stem cell field in recent time.

There is every reason to suppose that it will have widespread therapeutic applications for human diseases.

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